PATENT
Customer No. 22,852
Attorney Docket No. 09960.0002-01

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	)	
	)	
Keith Henry Stockman Campbell et al.	)	Group Art Unit: 1632
	)	
Serial No.: 09/225,233	)	Examiner: D. Crouch
	)	
Filed: January 4, 1999	)	Confirmation No. 2711
	)	

For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER

Mail Stop Appeal Brief--Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

TRANSMITTAL OF APPEAL BRIEF (37 C.F.R. 41.37)

Transmitted herewith is the APPEAL BRIEF in this application with respect to the Notice of Appeal filed on January 17, 2006.

This application is on behalf of

☐ Small Entity ☒ Large Entity

Pursuant to 37 C.F.R. 41.20(b)(2), the fee for filing the Appeal Brief is:

☐ $250.00 (Small Entity)

☒ $500.00 (Large Entity)

TOTAL FEE DUE:

Appeal Brief Fee	$500.00
Extension Fee (if any)	$1,710.00
Total Fee Due	$2,210.00

☒ Enclosed is a check for $2,210.00 to cover the above fees.

PETITION FOR EXTENSION. If any extension of time is necessary for the filing of this Appeal Brief, and such extension has not otherwise been requested, such an extension is hereby requested, and the Commissioner is authorized to charge necessary fees for such an extension to our Deposit Account No. 06-0916. A duplicate copy of this paper is enclosed for use in charging the deposit account.

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: June 16, 2006

By: _____

Salvatore J. Arrigo
Reg. No. 46,063
Tel: (202) 408-4160
Fax: (202) 408-4400
E-mail: arrigos@finnegan.com

PATENT
Customer No. 22,852
Attorney Docket No. 09960.0002-01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
Campbell et al.	) Group Art Unit: 1632
Application No.: 09/225,233	) Examiner: D. Crouch
Filed: January 4, 1999	) Confirmation No.: 2711
For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER	)

Attention: Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER BOARD RULE § 41.37

In support of the Notice of Appeal filed January 17, 2006, and further to Board Rule 41.37, Appellants present this brief and enclose herewith a check for the fee of $500.00 required under 37 C.F.R. § 1.17(c).

This Appeal Brief is being filed concurrently with a Petition for an Extension of Time for three months, and the appropriate fee.

This Appeal responds to the August 17, 2005, final rejection of claims 146-163.

Real Party In Interest

The real parties in interest are Start Licensing, Inc., of Austin, Texas, by virtue of a licensing agreement; Exeter Life Sciences, Inc., by virtue of a licensing agreement; Geron Corporation of Menlo Park, California, by virtue of a licensing agreement; and Roslin Institute (Edinburgh) of Midlothian, Great Britain, by virtue of an assignment from the inventors, an assignment from the Biotechnology & Biological Sciences Research Council, which was an assignee, and an assignment from Department for Environment, Food & Rural Affairs, London, Great Britain, having taken over the governmental functions of the Minister of Agriculture, Fisheries & Food, which was an assignee.

Related Appeals and Interferences

In accordance with 37 C.F.R. § 41.37(c)(1)(ii), appellant advises the Board of Patent Appeals and Interferences of the following pending appeals, interferences, or judicial proceedings which may be related to, directly affect, or be directly affected by or have a bearing on the Board's decision in the instant appeal: an appeal filed in U.S. application 09/658,862; and Interference Nos. 104,746 and 105,192, the decisions of which have been appealed under 35 U.S.C. § 146 as Case Nos. 1:05-cv-00353 RMU and 1:05-cv-00706-RMU in the United States District Court for the District of Columbia.

Status Of Claims

Claims 146-163 are rejected. The rejections of each of these claims is being appealed.

Status Of Amendments

No amendments have been filed subsequent to the final rejection on August 17, 2005. As previously agreed by appellants, Terminal Disclaimers were filed on March 31, 2006, over U.S. Patents 6,525,243; 6,147,276; and 6,252,133. Applicants held a telephonic interview with the Examiner on May 9, 2006, to discuss the Terminal Disclaimers. In the Interview Summary dated May 12, 2006, the Examiner indicated that the Terminal Disclaimers were approved, overcoming the obviousness-type double patenting rejections of record, and that applicants need not further address these rejections in the appeal brief.

Summary Of Claimed Subject Matter

Appellants were the first to create a clone, or copy, of a pre-existing mammal. (Specification at 34, Table 5.) Appellants' claims 146-154 are directed to a live-born clone of a pre-existing, non-embryonic, non-foetal, donor mammal produced by a process involving somatic cell nuclear transfer. Appellants' claims 155-163 are directed to a live-born clone of a pre-existing, non-embryonic, non-foetal, donor mammal.

Appellants' claimed clones have a number of unique features never seen in naturally occurring mammals or in mammals of the prior art. (*Id.* at 1, lines 6-14 and 28-33; at 6, lines 1-20; and at 19, lines 6-27.) These features are due the claimed animals being a clone, or copy, of a pre-existing mammal, which is created without normal sexual reproduction.

Mammals in nature are only generated through the process of normal sexual reproduction, which involves contributions from both of their biological parents. Thus,

these mammals are always a mixture of the genetic make-up of their two biological parents, and never have the same genetic complement as either of them.

Although animals had been previously created by "embryo cloning," in which an embryo was destroyed to generate multiple, identical mammals (*id.* at 1, lines 14-20), a mammal had never been generated that was a copy of a pre-existing, non-embryonic, non-foetal, donor mammal (*i.e.* "somatic cell cloning"). The difference between these two types of clones is that a clone generated by embryo cloning would not have the same genetic complement as either one of its parents. (*Id.*) This is due to the fact that the initial embryo could only be generated by normal mammalian sexual reproduction, which creates an animal that is a mixture of its two biological parents, and does not have the same genetic complement as either one of them. In contrast, appellants' clone is produced asexually. (*Id.* at 5, lines 5-8.) Thus, appellants' clone, which is generated by somatic cell cloning, would have the same genetic complement as one parent, which is referred to as the nuclear donor mammal. (*Id.* at 7, lines 13-20, and at 19, lines 6-27.)

Although appellants' claimed mammals are copies of a pre-existing, parental mammal, they are not the same mammal. (*Id.* at 1, lines 28-33, and at 19, lines 6-10.) They have phenotypic differences, occupy a different space, and exist during a different time than the parental mammal. (*Id.*)

Grounds of Rejection

A. Claims 146-163 stand ***provisionally*** rejected under 35 U.S.C. § 101 as allegedly claiming the same invention as that of claims 152-171 of copending

Application No. 09/658,862 because there is no distinction between appellants' claimed mammals and the mammals of claims 152-171 of Application No. 09/658,862.

B. Claims 146-163 stand rejected under 35 U.S.C. § 101 for allegedly being directed to non-statutory subject matter because appellants' claimed mammals are indistinguishable from mammals found in nature.

C. Claims 146-163 stand rejected under 35 U.S.C. § 112, first paragraph, because the specification allegedly does not enable clones of pre-existing mice, rabbits, horses, and rats.

D. Claims 146-163 stand rejected under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) for allegedly being anticipated by or obvious over several publications that teach embryo cloned cattle (Sims et al., 1993), sheep (McLaughlin et al., 1990), pigs (Prather et al., 1989), goats (Yong et al., 1991), mice (Cheong et al., 1993), and rabbits (Yang et al., 1992); standard bred horses (Lawrence et al., 1993); and laboratory rats (Gonzales-Pacheco et al., 1993).

Argument

A. Rejections under 35 U.S.C. § 101 for Statutory Double Patenting

The Examiner *provisionally* rejected claims 146-163 under 35 U.S.C. § 101 as allegedly claiming the same invention as that of claims 152-171 in copending Application No. 09/658,862. It is the Examiner's position that there is no distinction between the claimed mammals in the two applications. The Examiner is in error.

In order for appellants' claims to be invalid for statutory double patenting under 35 U.S.C. § 101, appellants must be claiming *identical subject matter* in pending claims 146-163 and claims 152-171 in copending Application No. 09/658,862. *See In re Vogel*,

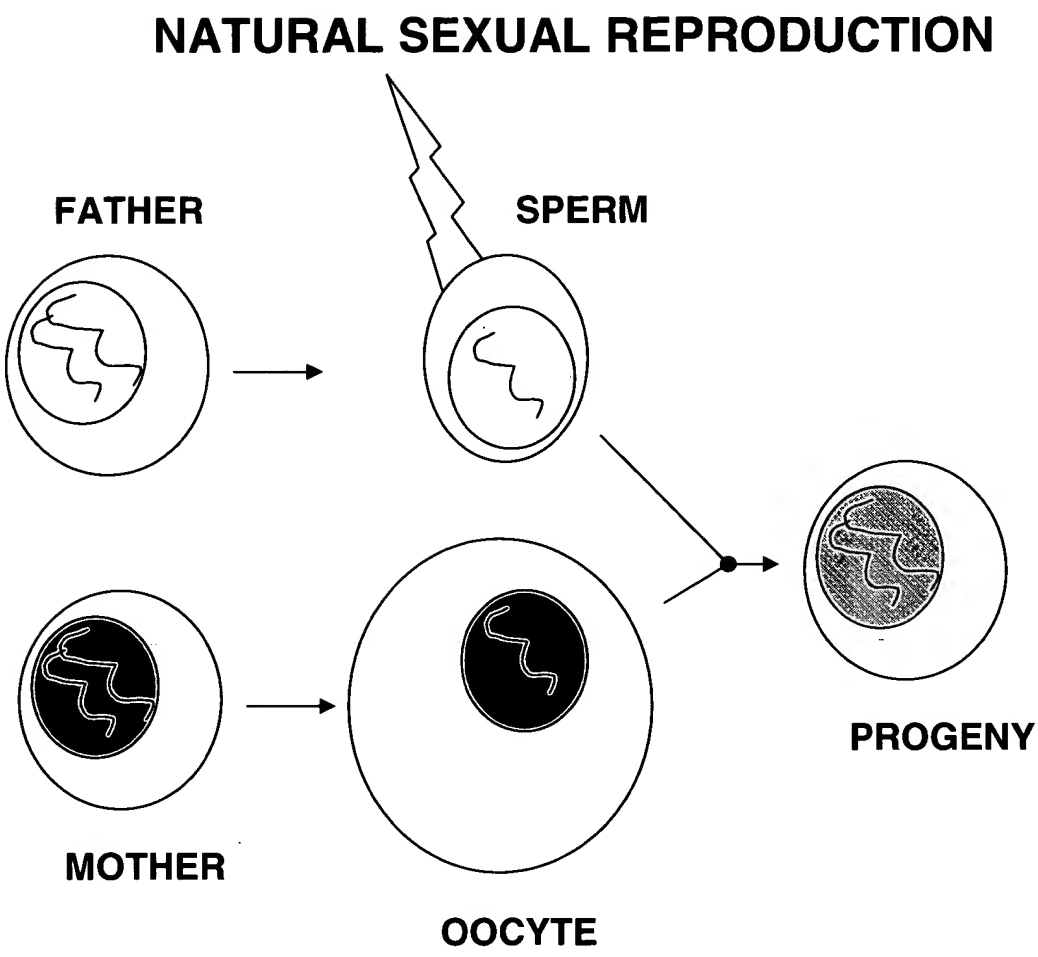
164 U.S.P.Q. 619, 621 (C.C.P.A. 1970). However, claims 146-163 in the instant application recite that the pre-existing mammal is a "non-foetal" mammal, whereas claims 152-171 in copending Application No. 09/658,862 do not contain this limitation. Thus appellants are not claiming *identical subject matter* in the two applications. Accordingly, applicants are not claiming the same invention as that of claims 152-171 in copending Application No. 09/658,862. For these reasons, the rejection of these claims statutory double patenting under 35 U.S.C. § 101 should be reversed.

B. Rejections under 35 U.S.C. § 101 for Claiming Non-Statutory Subject Matter

The Examiner also rejected claims 146-163 for allegedly being directed to non-statutory subject matter. It is the Examiner's position that appellants' claimed mammals are indistinguishable from mammals found in nature. The Examiner is in error. Appellants' clones are never found in nature. Thus, they are statutory subject matter.

1. **The mammals of claims 146-154 are never found in nature**

Claims 146-154 are directed to a live-born clone produced by a process involving somatic cell nuclear transfer. Nature never produces progeny by nuclear transfer. Rather, nature produces mammalian progeny by sexual reproduction, which appellant's show schematically below.

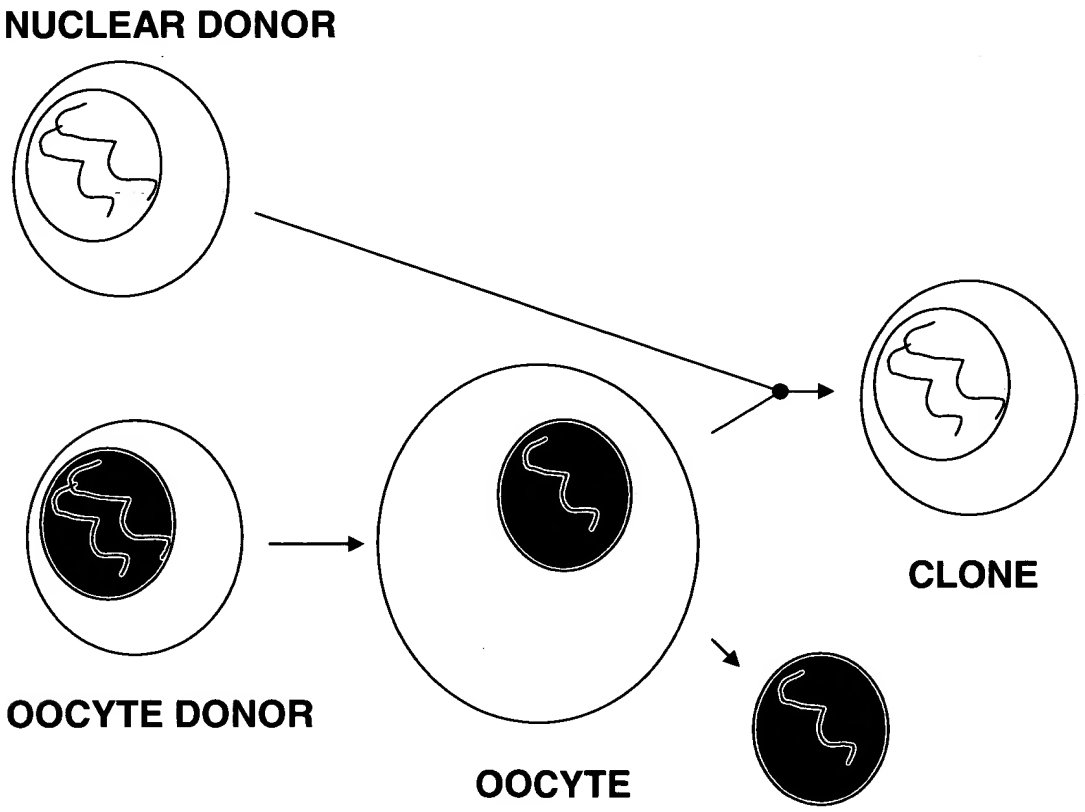


As is evident from this representation, mammalian sexual reproduction in nature requires two animals, a father and a mother animal, each with their distinct genetic chromosomal make-up. Sexual reproduction involves the joining of an oocyte (egg) and a sperm, each of which contains half of the normal chromosomal complement of the parents. When these two halves are combined to form the progeny mammal, the resulting progeny mammal contains a mixture of the chromosomal complement of the two parents. Thus, the progeny is not a copy of either parent. Consequently, nature

never makes a progeny mammal that is a copy of a single parental mammal -- sexual reproduction precludes such a result.

Somatic cell cloning by nuclear transfer results in a progeny mammal that is distinctly different from mammals produced in nature. This process is shown schematically below.

SOMATIC CELL CLONING



The process of somatic cell cloning results in a mammal having the same chromosomal complement as a single parent donor mammal. The animal that provides the nucleus is referred to as the nuclear donor animal. The oocyte (egg) is provided by the oocyte donor animal. The oocyte donor animal does not contribute to the nuclear

chromosomal content of the progeny animal in that the oocyte is "enucleated" to remove its nuclear genetic material. A complete complement of nuclear genetic material is then provided by the nuclear donor animal. The progeny clone receives its entire chromosomal complement from the donor mammal. In this way, the clone is a genetic copy of the nuclear donor mammal.

Since nature does not clone mammals by nuclear transfer, certainly the process steps recited in claims 146-154 do not occur in nature. Rather, it is evident that the hand of man must be involved in the process of claims 146-154. The Examiner is applying the wrong legal standard. (Office Action at 5-6.) As the Board noted in *Ex parte Allen*:

> The issue, in our view, in determining whether the claimed subject matter is patentable under Section 101 is simply whether that subject matter is made by man. If the claimed subject matter occurs naturally, it is not patentable subject matter under Section 101. . . The examiner has presented no evidence that the claimed polyploid oysters occur naturally without the intervention of man, nor has the examiner urged that polyploid oysters occur naturally. The record before us leads to no conclusion other than that the claimed polyploid oysters are non-naturally occurring manufactures or compositions of matter within the confines of patentable subject matter under 35 USC 101. Accordingly, the rejection under Section 101 must be reversed.

2 U.S.P.Q.2d 1425, 1426-7 (Bd. Pat. App. & Int. 1987). Thus, the legal standard to applied in the present case is whether appellants' clones occur naturally without the intervention of man. As in *Allen*, the Examiner has provided no evidence that appellants' claims would exist without the hand of man. Thus, as in *Allen*, the rejection under 35 U.S.C. § 101 must be reversed.

As the Supreme Court recognized in *Diamond v. Chakrabarty*, statutory subject matter includes "anything under the sun that is made by man." 206 U.S.P.Q. 193, 197

(1980). Appellants' claims require the intervention of man to perform the process step of nuclear transfer in claims 146-154. For these reasons, the rejection of these claims under 35 U.S.C. § 101 for claiming non-statutory subject matter should be reversed.

2. **The mammals of claims 155-163 are never found in nature**

Although claims 155-163 do not recite any process steps, these claims do require a ***clone*** of a pre-existing, non-embryonic, non-foetal, donor mammal. As discussed above, nature never makes clones of pre-existing non-embryonic, non-foetal, donor mammals. Rather, the clones of claims 155-163 must be made by man. As discussed above, the Examiner has provided no evidence that appellants' claims would exist without the hand of man. Thus, as in *Allen*, the rejection under 35 U.S.C. § 101 must be reversed. *See* 2 U.S.P.Q.2d at 1427.

As recognized by the Supreme Court in *Chakrabarty*, a non-naturally occurring animal is patentable subject matter under 35 U.S.C. § 101. *Id.* Since claims 155-163 require a non-naturally occurring animal, a clone, these claims are directed to statutory subject matter. For these reasons, the rejection of these claims under 35 U.S.C. § 101 for claiming non-statutory subject matter should be reversed.

C. Rejections under 35 U.S.C. § 112, first paragraph

The Examiner rejected claims 146-163 because the specification allegedly does not provide enablement for clones of mice, rabbits, horses, and rats. In each of these instances, the Examiner relies on papers reporting the ***successful*** cloning of these animals. This evidence is insufficient because it does not show that appellants' claimed process could not be used to successfully clone each of these species.

The Examiner's position ignores the general low efficiency of the cloning process. The cited papers simply show that there may be ways to increase the efficiency of cloning by optimization of appellants' invention. Any conclusions regarding the cloning of mammals must take into account the low efficiency of the cloning process. In view of this inefficiency of the cloning process, the fact that appellants' cloning process may have to be repeated many times to assure success does not mean that undue experimentation would be required. Rather, since any experimentation would be repetitive, it would be routine, and routine experimentation does not negate enablement. *See In re Wands*, 858 F.2d 731, 737; 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988).

There is no evidence of record that one of skill in the art would not succeed in using appellants' method to produce appellants' claimed mammals when the inefficiency of appellants' process is taken into consideration. In fact, the Office has not even alleged that appellants' claimed process will not work if nuclear transfer is performed a sufficient number of times. The fact that there may be ways to improve the efficiency of cloning through optimization of appellants' method does not negate that appellants' cloned mammals are enabled. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for appellants' claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1410, 1513 (Fed. Cir. 1993).

The Examiner concedes that clones of cattle, sheep, pigs, and goats are enabled. As detailed below, appellants' specification also enables cloned mice, rabbits, horses, and rats.

1. Claims 146 and 155

Claims 146 and 155 recite a Markush group of eight species, each of which is then recited separately in claims 147-154 and 156-163. Appellants argue for the enablement of these species separately below.

2. The Examiner concedes that claims 147-150 and 156-159 (cow, sheep, pig, and goat) are enabled

The Examiner conceded that clones of cattle, sheep, pigs, and goats are enabled. (August 17, 2005, Office Action 7.) Since claims 147-150 and 156-159 are directed to these species, the rejection of these claims should be reversed.

3. Claims 151 and 160 (mouse) are enabled

The Examiner's sole basis for questioning the enablement of mice is based on a paper reporting the ***successful*** cloning of mice using somatic cell nuclear transfer. (*See* Wakayama et al., 1998.) The Examiner takes the position that success in mice was achieved by including "a prolonged interval between nuclear injection and oocyte activation, suppressing cytokinesis." (August 17, 2005, Office Action at 8.)

The Examiner's position does not take into account the general low efficiency of the cloning process. As can be seen in Table 4 on page 34 of the specification, only 1 live lamb was born out of 277 "fused couplets" (i.e., oocytes with a transferred nucleus). Any conclusions regarding the cloning of mice must take into account the low efficiency of the cloning process. In view of this inefficiency of the cloning process, the fact that appellants' cloning process may have to be repeated many times to assure success in mice does not mean that undue experimentation would be required. Rather, since any experimentation would be repetitive, it would be routine, and routine experimentation

does not negate enablement. *See In re Wands*, 858 F.2d 731, 737; 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988).

There is no evidence of record that one of skill in the art would not succeed in using appellants' method to produce mice when the inefficiency of appellants' process is taken into consideration. In fact, the Office has not even alleged that appellants' claimed process will not work if nuclear transfer is performed a sufficient number of times. The fact that there may be ways to improve the efficiency of cloning through optimization does not negate that appellants' cloned mice are enabled. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for appellants' claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1410, 1513 (Fed. Cir. 1993).

In addition, according to the Examiner, successful cloning of mice, as reported by Wakayama, used a "prolonged interval between nuclear injection and oocyte activation." (August 17, 2005, Office Action at 8.) The Examiner further characterizes this "prolonged interval" as "1-6 hrs." (December 21, 2005, Advisory Action at 3.) Appellants' specification teaches such a prolonged interval between nuclear injection and oocyte activation on pages 11, lines 28-30: "where the recipient is an enucleated unactivated metaphase II oocyte, activation may take place ***subsequently***." The specification refers to application PCT/96/02098 as teaching this technique. (Specification at 12, lines 1-7). The Examiner concedes that the application discloses 6-20 hours for cows and that the time of activation is species dependent. (December 21, 2005, Advisory Action at 3.) Based on the teachings in appellants' specification, no

undue experimentation would have been required to arrive at Wakayama's 1-6 hour interval.

The specification further teaches an example of using enucleated unactivated metaphase II sheep oocytes on pages 22-24. Metaphase II oocytes were first recovered by flushing from the oviduct at 24-29 hours after hCG injection. (Specification at 22, lines 26-30.) Enucleation and nuclear transfer were next performed. (*Id.* at 23, lines 20-28.) Then, activation was induced at 32-34 hours post hCG injection. (*Id.* at 23, lines 32-33) Prior to activation, the couplets were washed, and then incubated in different media ***for more than 30 minutes***. (*Id.* at 23, lines 28-32.) Although this example does not provide the exact times that the oocytes were incubated prior to activation, it is clear from the specification this procedure used a prolonged interval, *i.e.*, more than 30 minutes, but less than 10 hours (*i.e.*, 34 hours - 24 hours), between nuclear injection and oocyte activation. The Examiner does not explain why this teaching is not sufficient to enable appellants' claims to mice.

For these reasons, the rejection of these claims under 35 U.S.C. § 112, first paragraph, should be reversed.

4. Claims 152 and 161 (rabbit) are enabled

The Examiner's sole basis for questioning the enablement of rabbits is based on a paper reporting the ***successful*** cloning of rabbits using somatic cell nuclear transfer. (*See* Chesne et al., 2002.) The Examiner takes the position that success in rabbits was achieved only when surrogate females were "asynchronous by 22 hours from recipient oocytes." (August 17, 2005, Office Action at 7.)

Once again, the Examiner's position does not take into account the general low efficiency of the cloning process. There is no evidence of record that one of skill in the art would not succeed in using appellants' method to produce rabbits when the inefficiency of appellants' process is taken into consideration. In fact, the Office has not even alleged that appellants' process will not work if nuclear transfer is performed a sufficient number of times. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for appellants' claimed invention.

The fact that there may be ways to improve this efficiency through optimization does not negate that appellants' cloned rabbits are enabled. While the cloning of rabbits, as reported by Chesne, used "asynchronous" embryo transfer, the use of asynchronous transfer with *in vitro* manipulated rabbit embryos was well-known prior to appellants' filing date. (Landa, 1981, and Al-Hasani et al., 1986.) Landa used synchronous and asynchronous embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen. (Landa at 265, Abstract.) Synchronous embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen, was not considered successful since only 1 of 94 of the embryos implanted. (*Id.* at 269, paragraph 1.) In contrast, ***asynchronous*** embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen, was more successful since 19 of 50 of the embryos implanted. (*Id.* at 269, paragraph 2.) Thus, it is not surprising that Chesne used "asynchronous" embryo transfer since Landa had shown that this technique was preferable with *in vitro* cultured embryos.

Al-Hasani et al. compared synchronous and asynchronous embryo transfer with *in vitro* cultured rabbit embryos. (Al-Hasani et al. at 187, Summary.) Al-Hasani et al. concluded that *in vitro* culture conditions lead to a delay in the development of embryos and that the longer the embryos are cultured *in vitro*, the greater the delay in the development of embryos. (*Id.* at 194, paragraph 1.) Al-Hasani et al. concluded that this delay in the development of embryos can be compensated, in part, by using an asynchronous recipient animal and provided optimal asynchronicities for embryos cultured *in vitro* for various periods of time. Asynchronicities in the recipients of minus 6 hours, minus 24 hours, and minus 36 hours are given as optimal for embryos cultured for different periods of time *in vitro*. (Al-Hasani et al. (1986) at 194, paragraph 1.) Chesne et al., relied on by the Examiner, used an asynchronicity of 22 hours.

Based on Landa (1981) and Al-Hasani et al. (1986), the benefit of using asynchronous transfer for *in vitro* manipulated rabbit embryos was known prior to appellants' effective filing date of August 1995, and the skilled artisan at that time would have expected that *in vitro* manipulations of rabbit embryos, such as those used in nuclear transfer, would lead to a delay in the development of rabbit embryos that could be compensated by asynchronous embryo transfer. Thus, the successful cloning of rabbits, as reported by Chesne, was accomplished using appellants' teachings together with a technique that was well-known in the art to improve the efficiency of rabbit embryo development. Appellants need not teach techniques that are well-known in the art.

For these reasons, the rejection of these claims under 35 U.S.C. § 112, first paragraph, should be reversed.

5. Claims 153 and 162 (horse) are enabled

The Examiner's sole basis for questioning the enablement of horses is based on a paper reporting the ***successful*** cloning of horses using somatic cell nuclear transfer. (*See* Galli et al., 2003.) The Examiner takes the position that success in horses "was aided by advances in assisted reproduction in the horse, including oocyte activation, when both protein synthesis and protein phosphorylation both must be inhibited[,] and zona-free manipulation." (August 17, 2005, Office Action at 8.)

Yet again, the Examiner's position does not take into account the general low efficiency of the cloning process. Once again, there is no evidence of record that one of skill in the art would not succeed in using appellants' method to produce horses when the inefficiency of appellants' process is taken into consideration. And again, the Office has not even alleged that appellants' process will not work if nuclear transfer is performed a sufficient number of times. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for appellants' claimed invention.

The fact that there may be ways to improve this efficiency through optimization does not negate that appellants' cloned horses are enabled. The successful cloning of horses, as reported in Galli et al., used procedures that were previously known to improve efficiencies of cloning prior to appellants' filing date. Galli cites to Lazari et al., *J. Reprod. Fertil. Abstr. Ser.* 28:73 (2002) for the proposition that both protein synthesis and protein phosphorylation must be inhibited. Lazari et al. does not support this proposition. Lazari et al. merely describes activation studies done in horses with the known protein synthesis inhibitor cycloheximide (CHX) and known protein

phosphorylation inhibitor DMAP, either alone or in combination, further in association with ionomycin. Lazari et al. report horse oocyte activation rates of 30.6% for CHX, 60% for DMAP, and 93% for CHX + DMAP. Thus, Lazari et al. simply shows that the combination increases the efficiency of cloning, not that appellants' method does not work.

Moreover, CHX and DMAP were well known compounds used in ooctye activation protocols prior to appellants' filing date. These activation protocols included sequential protocols where cellular Ca+ levels were initially reduced (for example, by electrical pulse or ionomycin) and then maintained at low levels (for example, by a protein synthesis inhibitor (CHX) or a phosphorylation inhibitor (DMAP)). *See e.g.*, Susko-Parrish et al., *Dev Biol.* 166(2):729-39 (1994). Thus, Lazari et al. showed that conventional activation protocols, which existed as of appellants' filing date, could be successfully employed with horse oocytes. The fact that they were used together does not detract from the fact that both compounds were well-known in the art for use in ooctye activation protocols, and that they would be expected to work together. Appellants need not teach techniques that are well-known in the art.

Galli also references Lagutino et al., *Thieronology* 59: 269 (2003) for evidence that the refinement of zona-free techniques aided their success. However, Lagutino et al. shows that zona-free manipulation is not critical for creating a horse embryo. Lagutino et al. compared the fusion rates of zona-intact and zona-free oocytes and subsequent cleavage rates of zona-intact and zona-free NT embryos. The observed rates were higher for zona-free, but zona-intact oocytes still had a 69% fusion rate and a 69% cleavage rate. Thus, Lagutino et al. confirms that conventional methods of

fusion, which were available to one of skill in the art as of appellants' filing date, were adequate to clone horses.

For these reasons, the rejection of these claims under 35 U.S.C. § 112, first paragraph, should be reversed.

6. Claims 154 and 163 (rat) are enabled

The Examiner's sole basis for questioning the enablement of rats is based on a paper reporting the ***successful*** cloning of rats using somatic cell nuclear transfer. (*See* Zhou et al., 1998.) The Examiner takes the position that success in rats was reached when "MG132, a protease inhibitor that reversibly blocks the first meiotic metaphase-anaphase transition in rat was used." (August 17, 2005, Office Action at 8.)

Yet again, the Examiner's position does not take into account the general low efficiency of the cloning process. There is no evidence of record that one of skill in the art would not succeed in using appellants' method to produce rats when the inefficiency of appellants' process is taken into consideration. In fact, the Office has not even alleged that appellants' process will not work if nuclear transfer is performed a sufficient number of times. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for appellants' claimed invention.

Moreover, the fact that there may be ways to improve this efficiency through optimization does not negate that appellants' cloned rats are enabled. The successful cloning of rats by Zhou involved the use of MG132, a protease inhibitor that reversibly blocks the first meiotic metaphase-anaphase transition. That is, the protease inhibitor MG132 helps maintain the oocytes in a **non-activated** state. This is just what the instant application teaches. The instant application specifically teaches reconstructing

an embryo using a ***non-activated*** MII arrested oocyte. (Specification at 4, last paragraph.) Thus, Zhou's successful cloning of rats was performed according to appellants' teachings.

For these reasons, the rejection of these claims under 35 U.S.C. § 112, first paragraph, should be reversed.

D. Rejections under 35 U.S.C. § 102(b)

The Examiner rejected claims 146-163 under 35 U.S.C. § 102(b) and/or 103(a) over several publications that teach embryo cloned cattle (Sims et al., 1993), sheep (McLaughlin et al., 1990), pigs (Prather et al., 1989), goats (Yong et al., 1991), mice (Cheong et al., 1993), and rabbits (Yang et al., 1992); standard bred horses (Lawrence et al., 1993); and laboratory rats (Gonzales-Pacheco et al., 1993). It is the Examiner's position that the mammals of the cited prior art anticipate appellants' claimed clones because appellants' claimed mammals do not exhibit a novel structural or functional difference from the prior art mammals. Essentially, the Examiner's argument is that, since appellants' clone is a copy of what previously existed, appellants' clone is anticipated by its parental donor mammal. The Examiner's position is in error because the prior art does not disclose animals that have all of the properties of appellants' claimed clones.

The issue in this appeal is whether the prior art discloses the ***identical invention*** as appellants' clone. *See Richardson v. Suzuki Motor Co. Ltd.*, 9 U.S.P.Q.2d 1913, 1920 (Fed. Cir. 1989). Anticipation is not shown by a ***non-identical*** prior art disclosure, even it if is "substantially the same" as the claimed invention. *See Jamesbury Corp. v. Litton Indust. Prod., Inc.*, 225 U.S.P.Q. 253, 256 (Fed. Cir. 1985). Because a mammal

identical to appellants' clone never existed before appellants' invention, the prior art cannot disclose appellants' claimed clone.

1. The specification explains that appellants' clone is not identical to the clone's parent

Appellants' specification explains why a clone is not identical to its parent:

> ***Animals produced by transfer of nuclei from a source of genetically identical cells share the same nucleus, but are not strictly identical as they are derived from different oocytes***. The significance of this different origin is not clear, but may affect commercial traits. Recent analyses of the mitochondrial DNA of dairy cattle in the Iowa State University Breeding Herd revealed [differences] associated with milk and reproductive performance (Freeman & Beitz, In Symposium on Cloning Mammals by Nuclear Transplantation (Seidel, G. E. Jr., ed.) 17-20, Colorado State University, Colorado (1992)). It remains to be confirmed that similar effects are present throughout the cattle population and to consider whether it is possible or necessary in specific situations to consider the selection of oocytes.

(Specification at 19, lines 7-21; emphasis added.)

Thus, appellants recognized that the claimed clone would have the same nucleus (i.e., chromosomal complement) as its parent, but that the clone and its parent would be different because of the fact that each is derived from a different oocyte. Since a different oocyte is used for the clone and its parent, appellants' clone and its parent cannot be ***identical***. For this reason, the parental mammal cannot anticipate appellants' clone.

2. The evidence of record demonstrates that appellants' clone is not identical to the clone's parent

The evidence provided to the Office during the prosecution of this application leaves no doubt that appellants' clone is not identical to its parent. As explained in Prather et al., 1990, cloned offspring may vary phenotypically due to environment and genetics. At 10, col. 2, 3rd full paragraph. With respect to genetic variation, DNA

rearrangements (as happens in the normal differentiation of immunoglobulins), gene amplifications, translocations, and dimunition are all possibilities of chromosomal rearrangements that would make a clone genetically different from its parent. *Id.*, at 10, col. 1, last full paragraph. In addition, differences in mitochondrial DNA (from the oocyte) may contribute to genetic differences between a clone and its parent. *Id.*, at 10, col. 1-2, bridging paragraph.

Moreover, environmental factors, such a uterine environment, generate differences that prevent a clone and its parent from being phenotypically identical. *Id.* at 10-11. Differences in phenotype may be manifested in many ways. For example, a clone and its parent may have phenotypic differences in color patterns caused by a different migration of melanocytes during development. Prather et al., 1990, at 10, 4th full paragraph, Wells et al., 1999, at 1003, col. 2, 2nd full paragraph. Similarly, since every iris is unique, a clone will have a different pigmentation of the iris as compared to its parent. U.S. Patent 4,641,349 at col. 4, lines 37-58. Thus, a clone that contains the same set of chromosomes as a single parental mammal can be distinguished from the parental mammal due to these environmental influences. February 5, 2003, Declaration of Dr. David Wells at ¶35.

Also, the cloned mammal will have behavioral differences from the parental mammal. *Id.*

Furthermore, appellants' claims require that the clone is of a pre-existing mammal. Thus, the cloned mammal will always be of a younger age than the parental mammal since the parental mammal must exist before the clone can exist. February 5,

2003, Declaration of Dr. David Wells at ¶35. As a result, the clone and the parental mammal cannot be ***identical***.

For example, appellants' specification describes the cloning of "Dolly" the sheep. Dolly was the first clone of a pre-existing, non-embryonic, non-foetal, donor mammal. Dolly existed at a later time than her parental donor mammal. In fact, the cell used to clone Dolly was taken from a six year old sheep. No one will ever confuse Dolly with her mother -- they are not identical sheep.

The evidence of record conclusively shows that appellants' clone is not identical to its parent due to genetic and phenotypic differences, including behavioral differences and a difference in the time periods in which the two animals exist. For these reasons, the parental mammal cannot anticipate appellants' clone. Similarly, none of the mammals of the cited references can anticipate appellants' claims. Appellants' claimed clones are produced by asexual reproduction, whereas all of the mammals of the cited references were produced by sexual reproduction. The rejection of these claims under 35 U.S.C. § 102(b) should be reversed.

E. Rejections under 35 U.S.C. § 103(a)

The Examiner rejected claims 146-163 under 35 U.S.C. § 103(a) over the same references as the rejections under 35 U.S.C. § 102(a). It is the Examiner's position that the mammals of the cited prior art make appellants' claimed clones obvious because there is no perceived structural or functional difference between the prior art mammals and appellants' claimed clones. Essentially, the Examiner's argument is that, since appellants' clone is a copy of what previously existed, appellants' clone is not different from its parent in a way that provides a patentable distinction. The Examiner's position

is in error because appellants' claimed clones contain non-obvious differences from their parental animals.

Appellants' clones have a feature that sets them apart from all of the animals in the prior art. Appellants' clones are *time-delayed copies* of pre-existing animals. This feature has two facets. First, appellants' clones are copies of the pre-existing parental mammal. This means that the clone has the same genetic complement as the parental mammal. Only a mammal cloned by somatic cell nuclear transfer will contain the same set of chromosomes as a single parental mammal. February 5, 2003, Declaration of Dr. David Wells at ¶34.

For this reason, a mammal cloned by somatic cell nuclear transfer is unlike any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell. *Id.* The set of chromosomes from any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, comes from two parental mammals, one male and one female. *Id.* In contrast, the set of chromosomes of a mammal cloned by somatic cell nuclear transfer is obtained from a single parental mammal. *Id.* This feature allows the cloned mammal to preserve the genetic information of the parental mammal without dilution. *Id.* Since somatic cell cloning was thought to be an impossibility prior to appellants' invention, this feature of appellants' clones cannot be considered to have been obvious over mammals produced by sexual reproduction.

In addition, appellants' clone has genetic and phenotypic differences from its parent, as discussed above. These differences are unknown until the clone is born.

Since what is unknown cannot be obvious, appellants' clone cannot be obvious over its parental mammal. *See In re Rijckaert*, 28 U.S.P.Q.2d 1955, 1957 (Fed. Cir. 1993).

Also, appellants' clone is time-delayed. Thus, appellants' cloned mammal will always be of a younger age than the parental mammal. This difference in the age of the cloned mammal could not have been expected from the prior art because, prior to appellants' invention, a mammal cloned by somatic cell nuclear transfer never existed. For example, "Dolly" the sheep existed at a later time than her parental donor mammal. No one would ever contend that the existence of Dolly the sheep was obvious in view of her mother. Prior to appellants' invention, no one could have ever had an expectation of success in producing such a mammal. Rather, somatic cell cloning was not thought to be possible at that time. Thus, appellants' clone is non-obvious over its parental mammal.

The evidence of record conclusively shows that appellants' clone has non-obvious differences from its parent, including a difference in the time periods in which the two animals exist. For these reasons, the rejection of these claims under 35 U.S.C. § 103(a) should be reversed.

Conclusion

For the reasons given above, pending claims 146-163 are allowable and reversal of the Examiner's rejection is respectfully requested.

To the extent any extension of time under 37 C.F.R. § 1.136 is required to obtain entry of this Appeal Brief, such extension is hereby respectfully requested. If there are any fees due under 37 C.F.R. §§ 1.16 or 1.17 which are not enclosed herewith, including any fees required for an extension of time under 37 C.F.R. § 1.136, please charge such fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: June 16, 2006

By: _____

Salvatore J. Arrigo
Reg. No. 46,063
Tel: (202) 408-4160
Fax: (202) 408-4400
email: arrigos@finnegan.com

Claims Appendix to Appeal Brief Under Rule 41.37(c)(1)(viii)

The claims on appeal are:

146. A live-born clone of a pre-existing, non-embryonic, non-foetal, donor mammal from which a differentiated cell has been taken,

wherein the mammal is selected from cattle, sheep, pigs, goats, mice, rabbits, horses, and rats,

wherein the clone is produced by a process comprising:

(a) transferring the nucleus of a somatic cell of the non-embryonic, non-foetal, mammal or a cell obtained by culture thereof into a suitable enucleated recipient cell from the same species,

wherein the somatic cell or cell obtained by culture thereof is a quiescent diploid cell at the time of transfer;

(b) activating the recipient cell before, during, or after nuclear transfer;

(c) incubating the reconstituted cell such that an embryo develops;

(d) transferring the embryo to a female of the same species; and

(e) developing the embryo into the live-born clone.

147. The clone of claim 146, wherein the mammal is a cattle.

148. The clone of claim 146, wherein mammal is a sheep.

149. The clone of claim 146, wherein the mammal is a pig.

150. The clone of claim 146, wherein the mammal is a goat.

151. The clone of claim 146, wherein the mammal is a mouse.

152. The clone of claim 146, wherein the mammal is a rabbit.

153. The clone of claim 146, wherein the mammal is a horse.

154. The clone of claim 146, wherein the mammal is a rat.

155. A live-born clone of a pre-existing, non-embryonic, non-foetal, donor mammal, wherein the mammal is selected from cattle, sheep, pigs, goats, mice, and rabbits, horses, and rats.

156. The clone of claim 155, wherein the mammal is a cattle.

157. The clone of claim 155, wherein mammal is a sheep.

158. The clone of claim 155, wherein the mammal is a pig.

159. The clone of claim 155, wherein the mammal is a goat.

160. The clone of claim 155, wherein the mammal is a mouse.

161. The clone of claim 155, wherein the mammal is a rabbit.

162. The clone of claim 155, wherein the mammal is a horse.

163. The clone of claim 155, wherein the mammal is a rat.

Evidence Appendix to Appeal Brief Under Rule 41.37(c)(1)(ix)

Wakayama et al., 1998. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Chesne et al., 2002. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Landa, 1981. Filed by appellants: November 16, 2005. Entered by Examiner: December 12, 2005, Office Action.

Al-Hasani et al., 1986. Filed by appellants: November 16, 2005. Entered by Examiner: December 12, 2005, Office Action.

Galli et al., 2003. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Lazari et al., *J. Reprod. Fertil. Abstr. Ser.* 28, 73 (2002). Filed by appellants: November 16, 2005. Entered by Examiner: December 12, 2005, Office Action.

Susko-Parrish et al., *Dev Biol.* 1994 Dec;166(2):729-39. Filed by appellants: November 16, 2005. Entered by Examiner: December 12, 2005, Office Action.

Lagutino et al., *Thieronology.* 59, 269 (2003). Filed by appellants: November 16, 2005. Entered by Examiner: December 12, 2005, Office Action.

Zhou et al., 1998. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Sims et al., 1993. Cited by Examiner: PTO-892 attached to Paper No. 19 (January 30, 2002).

McLaughlin et al., 1990. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Prather et al., 1989. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Yong et al., 1991. Cited by Examiner: PTO-892 attached to Paper No. 19 (January 30, 2002).

Cheong et al., 1993. Cited by Examiner: PTO-892 attached to Paper No. 19 (January 30, 2002).

Yang et al., 1992. Cited by Examiner: PTO-892 attached to Paper No. 19 (January 30, 2002).

Lawrence et al., 1993. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Gonzales-Pacheco et al., 1993. Cited by Examiner: PTO-892 attached to Paper No. 300 (August 17, 2005).

Prather et al., Nuclear transplantation as a method for cloning embryos, *Proc Soc Exp Biol Med.* 1990 Oct;195(1):7-12. Filed by appellants: July 1, 2002. Entered by Examiner: October 9, 2002, Office Action.

Wells et al., Production Of Cloned Calves Following Nuclear Transfer With Cultured Adult Mural Granulosa Cells, *Biology of Reproduction* 60:996-1005 (1999). Filed by appellants: July 1, 2002. Entered by Examiner: October 9, 2002, Office Action.

U.S. Patent 4,641,349 to Flom et al. Filed by appellants: July 1, 2002. Entered by Examiner: October 9, 2002, Office Action.

Declaration of Dr. David Wells. Filed by appellants: February 10, 2003. Entered by Examiner: May 5, 2003, Office Action.

gradients close to the air–water interface, where veils develop within a few minutes. A suspension of latex beads (1 or 0.5 μm) was then added with a capillary pipette. Preparations were recorded with dark-field illumination using a CCD camera (Sony) attached to the microscope and a video recorder. Afterwards, positions of individual latex beads were recorded frame by frame (at 0.04-s intervals). Only trajectories that were almost parallel to the plane of observation were included.

Oxygen gradients. Oxygen microelectrodes (with a 5–10-μm tip)[3] connected to a picoammeter were mounted in a micromanipulator. The surface of the *Thiovulum* veil and the electrode tip were observed through a dissection microscope tilted at an angle of 45°; this made it possible to map the O_2 isopleths inside and above the veil. Microelectrodes penetrating from above tend to deform the O_2 diffusion gradients above the sediment[14], but this should not affect the relative position of O_2 isopleths in different areas of the veil. The fact that O_2 was measured in a vertical convective flow is also likely to minimize this effect.

Received 27 January; accepted 8 May 1998.

1. Purcell, E. M. Life at low Reynolds number. *Am. J. Phys.* 45, 3–11 (1977).
2. Revsbech, N. P., Jørgensen, B. B. & Blackburn, T. H. Oxygen in the sea bottom measured with a microelectrode. *Science* 207, 1355–1356 (1980).
3. Revsbech, N. P. & Jørgensen, B. B. Microelectrodes: their use in microbial ecology. *Adv. Microbiol. Ecol.* 9, 293–352 (1986).
4. Wirsen, C. O. & Jannasch, H. W. Physiological and morphological observations on *Thiovulum* sp. *J. Bacteriol.* 136, 765–774 (1978).
5. Garcia-Pichel, F. Rapid bacterial swimming measured in swarming cells of *Thiovulum majus*. *J. Bacteriol.* 171, 3560–3563 (1989).
6. Fenchel, T. Motility and chemosensory behaviour of the sulphur bacterium *Thiovulum majus*. *Microbiology* 140, 3109–3116 (1994).
7. Jørgensen, B. B. & Revsbech, N. P. Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp. in O_2 and H_2S microgradients. *Appl. Environ. Microbiol.* 45, 1261–1270 (1983).
8. Lighthill, J. Flagellar hydrodynamics. *Soc. Indust. Appl. Math. Rev.* 18, 161–230 (1976).
9. Vogel, S. *Life in moving fluids* (Grant, Boston, 1981).
10. Fenchel, T. & Bernard, C. Mats of colourless sulphur bacteria. I Major microbial processes. *Mar. Ecol. Prog. Ser.* 128, 161–170 (1995).
11. Aller, R. C. & Yingst, J. Y. Effects of marine deposit-feeders *Heteromastus filiformis* (Polychaeta), *Macoma balthica* (Bivalvia), and *Tellina texana* (Bivalvia) on averaged solute transport, reaction rates, and microbial distributions. *J. Mar. Res.* 41, 299–322 (1985).
12. Dando, P. R. *et al.* The effects of methane seepage at an intertidal/shallow subtidal site on the shore of Kattegat, Vendsyssel. *Bull. Geol. Soc. Denmark* 41, 65–79 (1994).
13. Gundersen, J. K., Jørgensen, B. B., Larsen, E. & Jannasch, H. W. Mats of giant sulphur bacteria on deep-sea sediments due to fluctuating hydrothermal flow. *Nature* 360, 454–455 (1992).
14. Glud, R. N., Gundersen, J. K., Revsbech, N. P. & Jørgensen, B. B. Effects on the benthic diffusive boundary layer imposed by microelectrodes. *Limnol. Oceanogr.* 39, 462–467 (1994).

Acknowledgements. We thank J. Johansen for technical assistance. This study was supported by grants from the Danish Natural Science Research Council.

Correspondence and requests for materials should be addressed to T.F. (e-mail: mbltf@inet.uni2.dk).

Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei

T. Wakayama*†, A. C. F. Perry*‡, M. Zuccotti*§, K. R. Johnson‖ & R. Yanagimachi*

* *Department of Anatomy and Reproductive Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822, USA*
† *Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*
‡ *Department of Signalling, Babraham Institute, Cambridge CB2 4AT, UK*
§ *Dipartimento Biologia Animale, Laboratorio Biologia dello Sviluppo, University of Pavia, Piazza Botta 10, 27100, Pavia, Italy*
‖ *Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA*

Until recently, fertilization was the only way to produce viable mammalian offspring, a process implicitly involving male and female gametes. However, techniques involving fusion of embryonic or fetal somatic cells with enucleated oocytes have become steadily more successful in generating cloned young[1–3]. Dolly the sheep[4] was produced by electrofusion of sheep mammary-derived cells with enucleated sheep oocytes. Here we investigate the factors governing embryonic development by introducing nuclei from somatic cells (Sertoli, neuronal and cumulus cells) taken from adult mice into enucleated mouse oocytes. We found that some enucleated oocytes receiving Sertoli or neuronal nuclei developed *in vitro* and implanted following transfer, but none developed beyond 8.5 days post coitum; however, a high percentage of enucleated oocytes receiving cumulus nuclei developed *in vitro*. Once transferred, many of these embryos implanted and, although most were subsequently resorbed, a significant proportion (2 to 2.8%) developed to term. These experiments show that for mammals, nuclei from terminally differentiated, adult somatic cells of known phenotype introduced into enucleated oocytes are capable of supporting full development.

Previous studies have suggested that embryonic development is enhanced when donor nuclei are in the G0 or G1 phase of the cell cycle[1,3,4], and Dolly the sheep developed from an enucleated oocyte electrofused with a mammary-derived cell presumed to be in G0 following culture in serum-deficient medium for 5 days[4]. We have investigated the developmental potential of oocytes injected with the nuclei of non-cultured cells known to be at G0. We selected

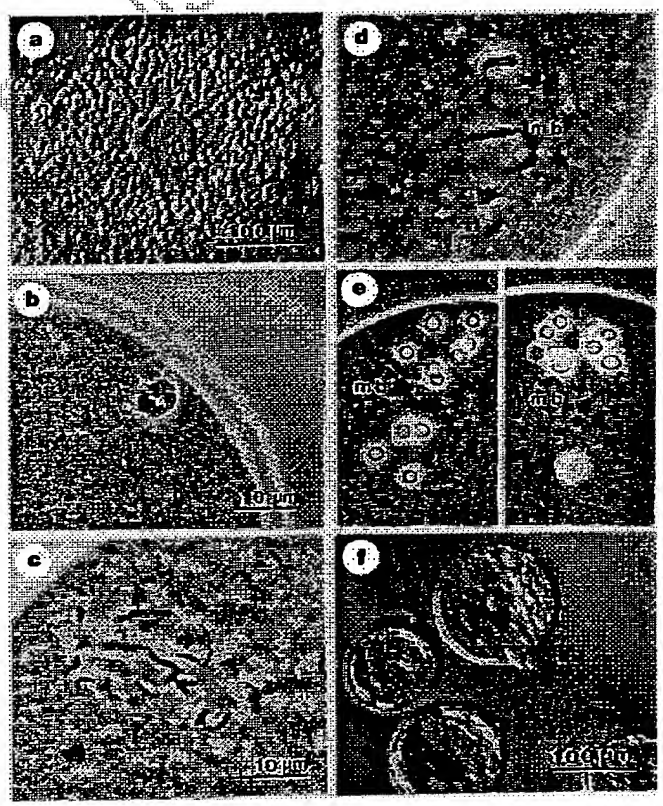


Figure 1 *In vitro* development of enucleated oocytes following injection of cumulus cell nuclei. **a**, Live oocyte surrounded by cumulus cells. The egg coat (the zona pellucida) appears in this micrograph as a relatively clear zone around the oocyte. **b–e**, Behaviour of cumulus cell nuclei following injection into enucleated oocytes, photographed after fixation and staining. **b**, A cumulus cell nucleus within 10 min of injection. **c**, Transformation of the nucleus into disarrayed chromosomes 3 h after injection. The disorder reflects an unusual situation in which single, condensed chromatids are each attached to a single pole of the spindle and are therefore not aligned on a metaphase plate. **d**, 1 h after Sr^{2+} activation, chromosomes are segregated into two groups (mb, midbody). **e**, 5 h after Sr^{2+} activation, two pseudo-pronuclei (left and right panels) with a varying number of distinct nucleolus-like structures are discernible in each egg. The size and number of pseudo-pronuclei varied, suggesting that segregation of chromosomes was random after oocyte activation. **f**, Live blastocysts produced following injection of enucleated oocytes with cumulus cell nuclei.

Table 1 Preimplantation of enucleated eggs injected with cumulus cell nuclei

Time of oocyte activation	Total no. of oocytes used	No. of enucleated oocytes	No. of surviving oocytes after injection	No. (%) of activated oocytes	No. (%, mean ± s.d.) of embryos developed from oocytes at 72 h after activation		
					1-cell and abnormal	2-8-cell	Morula/blastocyst*
Simultaneously with injection	233	230	182	153 (84.1)	17	75	61 (39.9 ± 16.6)
1-3 h after injection	573	565	508	474 (93.3)	20	177	277 (58.4 ± 12.6)
3-6 h after injection	195	191	182	151 (83.0)	9	41	101 (66.9 ± 14.4)

*There is a significant different ($P < 0.005$) between the top result and the bottom two. Data were analysed using the χ^2 test.

Sertoli, neuronal and cumulus from adult mice as representatives of this class; Sertoli cells and neurons do not normally divide in adults but remain at G0, and more than 90% of cumulus cells surrounding recently ovulated oocytes (Fig. 1a) are in the G0/G1 phase of the cell cycle[5]. These somatic cell types have very distinctive morphologies, making them easy to identify with confidence. All cells were used immediately (that is, without *in vitro* culturing) following the removal of tissue from freshly killed mice.

Enucleated mouse oocytes were each injected with a single nucleus from one of the three somatic cell types and left for 0 to 6 hours before activation. Examination of enucleated oocytes injected with cumulus nuclei revealed that chromosome condensation had occurred within 1 hour of injection (Fig. 1b, c). When, after 1 to 6 hours incubation, oocytes were activated in culture medium containing Sr^{2+} and cytochalasin B, their cumulus-derived chromosomes segregated (Fig. 1d) to form structures resembling the pronuclei that are formed after normal fertilization (referred to here as pseudo-pronuclei). Examination of 47 such oocytes after fixation and staining showed that 64% had two pseudo-pronuclei (Fig. 1e) and 36% had three or more. Oocytes with distinct pseudo-pronuclei were considered to be activated. Owing to the cytokinesis-blocking effect of cytochalasin B, no polar body was formed and therefore all chromosomes were retained within the oocyte, regardless of the number of pseudo-pronuclei. Chromosome analysis of 13 such oocytes fixed before the first cleavage (data not shown) revealed that 85% had a normal total chromosome number ($2n = 40$). The time interval between nucleus injection and oocyte activation appeared to affect the rate of oocyte development (Table 1). Activation immediately after nucleus injection led to

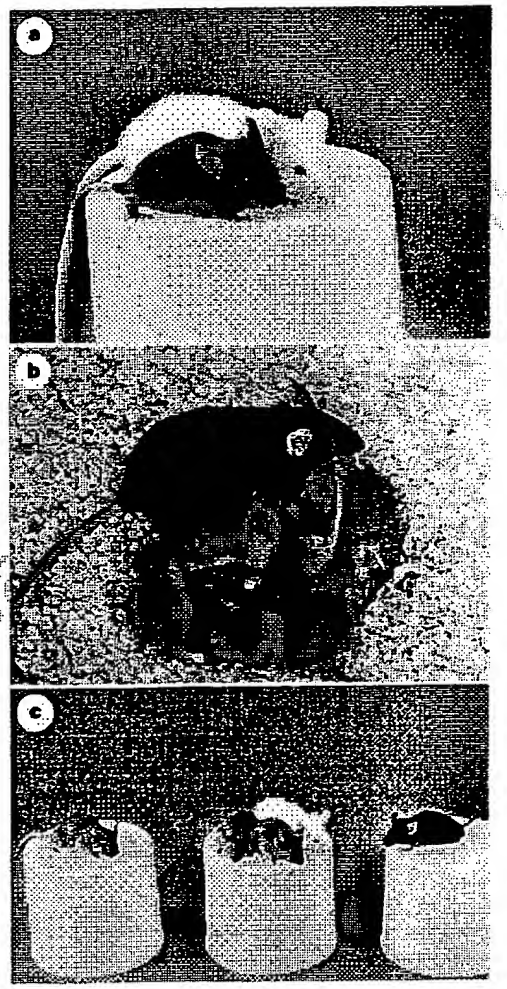


Figure 2 Cloned mice. **a**, The first surviving cloned mouse, Cumulina (born 3 October 1997) at four weeks (foreground) with her foster mother. **b**, Cumulina at 2.5 months with the pups she produced following mating with a CD-1 (albino) male. **c**, Two B6C3F1-derived, cloned, agouti young (centre) in front of their albino foster mother (CD-1), and a B6D2F1 oocyte donor (black, right). The two agouti offspring in the centre are clones (identical 'twin' sisters) of the agouti B6C3F1 cumulus donor shown on the left, and are two of the offspring described in series C (see text) and Table 2.

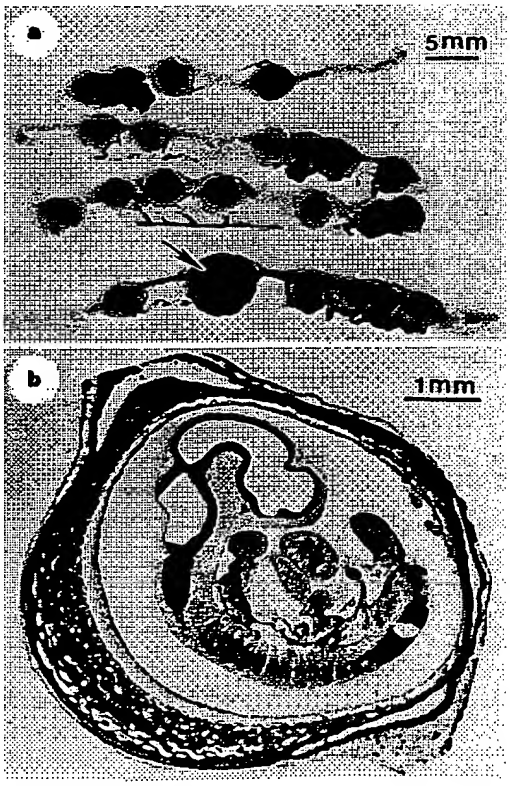


Figure 3 Development following uterine transfer of embryos produced after injection of Sertoli cell nuclei into enucleated oocytes. **a**, Uteri of recipient females 8.5 d.p.c., fixed with Bouin's fluid, dehydrated and cleared with benzyl benzoate. All uterine implantation sites failed to develop except for one (arrow), in which an embryo (**b**) appeared to be normal and was at the ~12-somite stage.

Table 2 Development of enucleated eggs injected with Sertoli or brain-cell nuclei*

Cell type injected	No. of surviving oocytes injected	No. (%) of oocytes activated	Total no. (%) of morulae/blastocysts developed†	No. of transferred embryos (recipients)	No. (%) of implantation sites	No. (%) of fetuses
Sertoli	159	159 (100)	63 (39.6)	59 (8)	41 (69.5)	1 (1.7)
Brain	228	223 (97.8)	50 (22.4)	46 (5)	25 (54.3)	1 (2.2)‡

* All recipients were killed at 8.5 d.p.c.
† There is a significant difference ($P < 0.005$) between the top and bottom result.
‡ Died at about 6–7 d.p.c.

significantly poorer development to morulae/blastocysts *in vitro* (Fig. 1f) than was achieved when activation followed a delay of 1 to 6 hours.

On the basis of this information, we injected Sertoli and neuronal nuclei into enucleated oocytes and delayed activation for 1 to 6 hours: about 40% of enucleated oocytes that had been injected with Sertoli cell nuclei, and 22% of those injected with neuronal nuclei, developed into morulae/blastocysts *in vitro* (Table 2). As these values were less than those achieved after cumulus nucleus injection (58–67%; Table 1), we concentrated on the potential of cumulus cell nuclei to support embryonic development *in vivo*.

In the first series of experiments (series A in Table 3), a total of 142 developing embryos (at the 2-cell to blastocyst stage) were transferred to 16 recipient females. When these females were examined at 8.5 and 11.5 d.p.c., 5 live and 5 dead fetuses were seen *in uteri*. In the second series (series B in Table 3), a total of 800 embryos were transferred into 54 foster mothers, and caesarean sections at 18.5–19.5 d.p.c. revealed 17 live fetuses. Of these, six died soon after delivery, one died approximately 7 days after delivery, but the remaining ten females survived and are apparently healthy. All of these, including the first-born survivor (born on 3 October 1997 and named 'Cumulina'; Fig. 2a), have been mated and have delivered and raised normal offspring (Fig. 2b). Several of these offspring have, in turn, now developed into fertile adults.

In the third series of experiments (series C in Table 3), B6C3F1 mice carry a copy of the *agouti* (*A*) gene, and are consequently agouti; offspring from this experiment should therefore have an agouti coat colour, rather than the black of the B6D2F1 oocyte donors. A total of 298 embryos derived from B6C3F1 cumulus cell nuclei were transferred to 18 foster mothers. Caesarean sections at 19.5 d.p.c. revealed six live fetuses whose placentas were used in DNA-typing analysis. Although one died a day after birth, the five extant females are healthy and have the agouti coat phenotype. Figure 2c shows two such agouti pups with their albino foster mother (CD-1).

We did additional experiments (series D in Table 3) to investigate whether clones could be more efficiently cloned in subsequent rounds of recloning. We therefore collected cumulus cells from B6C3F1 (agouti) clones generated in series C and injected their nuclei into enucleated B6D2F1 oocytes to generate embryos that were transferred as described for series B and C. A total of 287 embryos derived from cloned B6C3F1 cumulus-cell nuclei were transferred to 18 foster mothers. When caesarean sections were done at 19.5 d.p.c. eight live fetuses were recovered. Although one died soon after birth, the seven surviving females are healthy and have the predicted agouti coat phenotype. These results indicate that clones (series B and C) and cloned clones (series D) are produced with comparable efficiency. This argues that successive generations of clones do not undergo changes (either positive or negative) that influence the outcome of the cloning process.

We also monitored the developmental potential *in vivo* of morulae/blastocysts generated following the injection of either Sertoli-cell or neuronal nuclei into enucleated oocytes (all cells from non-clones) (Table 2). Embryos produced by Sertoli-nucleus injection resulted in a single live fetus (Fig. 3) in the uterus of a foster mother killed 8.5 d.p.c. (Table 2). We failed to detect *in vivo* development of embryos derived following injection of neuronal nuclei beyond 6–7 d.p.c.

We believe that all of the live offspring reported here represent clones derived from cumulus-cell nuclei in the absence of genetic

Table 3 Postimplantation development of enucleated eggs injected with cumulus cell nuclei

Experiment series*	Time of oocyte activation	No. of injected oocytes	No. of transferred embryos (recipients)	No. (%) of implantations from transferred embryos†	No. of fetuses developed from transferred embryos					No. (%) of newborn from transferred embryos
					Total (%)†	8.5 d.p.c.		11.5 d.p.c.		
						Live	Dead	Live	Dead	
A	Simultaneously with injection	82	34 (4)	8 (23.5)	0					–
	1–3 h after injection	136	45 (5)	32 (71.1)	7 (15.6)	3	2‡	2	0	–
	3–6 h after injection	124	63 (7)	36 (57.1)	3 (4.8)	0	2§	0	1‖	–
B	1–3 h after injection	1345	760 (49)	–	–	–	–	–	–	16 (2.1)
	3–6 h after injection	62	40 (5)	–	–	–	–	–	–	1 (2.5)
C	1–3 h after injection	458	298 (18)	–	–	–	–	–	–	6 (2.0)
D	1–3 h after injection	603	287 (18)	–	–	–	–	–	–	8 (2.8)

* Series A, caesarean sections were done at 8.5 or 11.5 d.p.c.; series B and C, caesarean sections were done at 18.5–19.5 d.p.c. In series A and B, each donor nucleus is from a B6D2F1 cumulus cells; in series C, each donor nucleus is from a B6C3F1 cumulus cell; in series D, each donor nucleus is from a B6C3F1 clones mouse from series C.
† There is a significant difference between the top result and the bottom two: implantation ($P < 0.005$); fetal development ($P < 0.05$). Data were analysed by χ^2 tests.
‡ Died 6–7 d.p.c.
§ Died 7–8 d.p.c.
‖ Died 10 d.p.c.

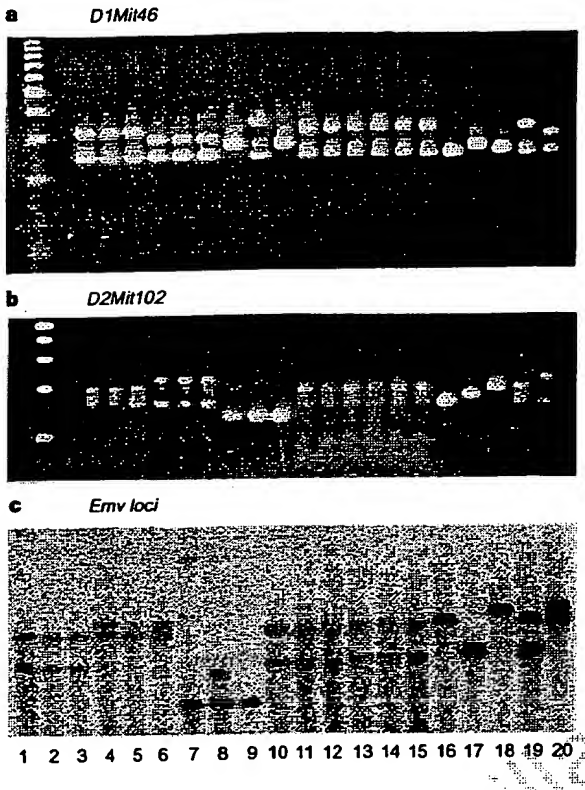


Figure 4 DNA typing of donors and offspring in series C corroborates the genetic identify of the cloned offspring to cumulus cell donors, and non-identity to oocyte donors and host foster females. **a**, PCR typing using the strain-specific marker *D1Mit46*. **b**, PCR -amplified DNA (**a**, **b**) from F_1 hybrid mice gives an additional gel band not seen in the DNA from inbred parental strains (lanes 16-20); this extra band corresponds to a heteroduplex derived from the two parental products, whose conformation results in anomalous gel migration. **c**, Southern blot typing of strain-specific *Emv* loci (*Emv1*, *Emv2* and *Emv3*). Placental DNA from the six cloned series C offspring (lanes 10-15) was compared with DNA from the three cumulus cell donor females (lanes 1-3), the three oocyte recipient females (lanes 4-6), and the three host females (lanes 7-9). Control DNA was from C57BL/6 (lane 16), C3H (lane 17), DBA/2 (lane 18), B6C3F1 (lane 19) or B6D2F1 (lane 20). 100-bp DNA size-marker ladders are shown on the left of **a** and **b**.

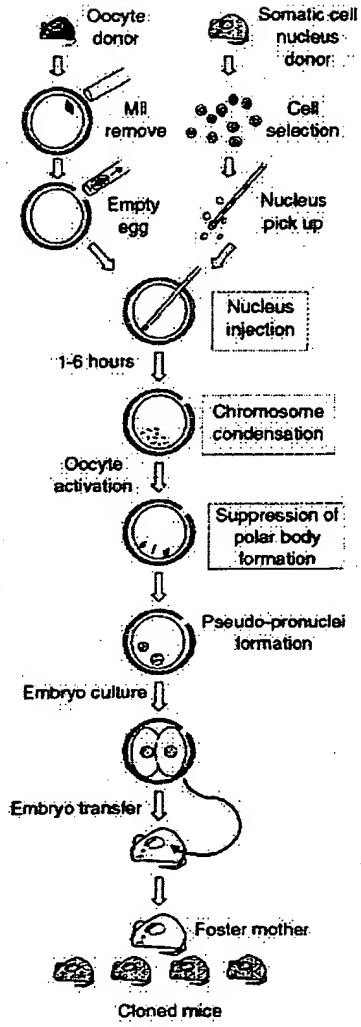


Figure 5 The cloning procedure developed here, as described in the text and Methods section.

contamination, for the following reasons. (1) Oocytes/eggs were not exposed to spermatozoa *in vitro*. (2) Foster mothers (CD-1, albino) were mated with vasectomized males (CD-1, albino) of established infertility. In the unlikely event of fertilization by such a vasectomized male, the offspring would be albino. We transferred 2- to 8-cell embryos/blastocysts into the oviducts/uteri of foster mothers; it is well established that 2- to 8-cell mouse embryos/blastocysts cannot be fertilized by spermatozoa[6]. (3) All full-term animals were born with black eyes; the surviving ten from series B have black coats and the surviving five in series C have agouti coats. This pattern of coat colour inheritance exactly matches that predicted by the genotype of the nucleus donor in each case. As B6D2F1 mice lack the *agouti* gene, the agouti mice in series C must have inherited their agouti coat colour from a non-B6D2F1 nucleus. (4) Where possible, all putative clones have been sexed, and all were found to be females, consistent with their genetic progenitors invariably being female. (5) DNA typing of highly variable alleles diagnostic of the B6, C3, D2 and CD-1 strains used here (Fig. 4) demonstrates beyond reasonable doubt that the six cloned offspring in series C (which includes one that died soon after birth) are isogenic with the three cumulus cell donor females used (B6C3F1) and do not contain DNA derived from either the oocyte donors (B6D2F1) or host foster mothers (CD-1). (6) Following enucleation, we suppressed extrusion of chromosomes into polar bodies using cytochalasin B. Thus, even if enucleation of the oocytes had been either totally unsuccessful or only partly successful, all resulting zygotes would be hyperploid; such embryos are inviable and cannot develop into normal offspring[7]. Moreover, in mock experiments, we enucleated 204 oocytes and examined them after fixation and staining[8]: no chromosomes were apparent, suggesting that the efficiency of chromosome removal exceeded 99.99%.

In general, nuclei have previously been transferred either into enucleated, one-cell embryos[9], or into unfertilized, enucleated oocytes[10,11] which were then immediately activated, thereby preventing chromosome condensation[12]. Mouse embryonic stem[13] or primordial germ[14] cell nuclei transferred into enucleated oocytes that were then immediately activated produced embryos capable of developing to blastocysts, with a minority of these implanting. In contrast, activation that was delayed for 30 to 60 minutes after the introduction of thymocyte nuclei into enucleated oocytes[15] often resulted in the extrusion of a pseudo-polar body, with a consequently high incidence of hypoaneuploidy (78%) and with none of the embryos developing beyond the 4-cell stage.

We have shown that a relatively high proportion of enucleated oocytes can develop to morulae/blastocysts and beyond when they are activated after a prolonged delay following injection of adult-

derived, somatic cell nuclei. Indeed, the inclusion of a prolonged interval between nuclear injection and oocyte activation (and suppression of cytokinesis) was apparently beneficial for both pre- and post-implantation development (Tables 1, 3). Although this seems paradoxical after earlier work, prolonged exposure of incoming nuclei to a cytoplasm rich in metaphase promoting factor causes persistent chromosome condensation (in the absence of DNA synthesis) and may facilitate the nuclear changes that are essential for development. We are studying the molecular events attending this latent period with respect to potential epigenetic 'reprogramming' and chromatin repair, *inter alia*. Also, the use of a piezo-impact pipette drive unit[16,17] may have contributed to a high rate of embryonic development by enabling oocyte and donor nucleus manipulation to be quick and efficient, thereby reducing the trauma to both in comparison with methods using electrofusion, Sendai virus or polyethylene glycol. Furthermore, we minimized the amount of somatic cell cytoplasm introduced into enucleated oocytes, which might otherwise have interfered with the onset of development.

It is unclear why Sertoli and neuronal cell nuclei failed to produce full-term embryos. Although our study does not preclude the possibility that these nuclei (and nuclei from other cell types) may be able to support full-term development, this finding suggests that the G0 status of donor nuclei is not sufficient *per se* to ensure embryonic development. We did not use mural granulosa cells, which differ functionally and in their subsequent fate from cumulus cells[18], and the question is open as to whether their nuclei might prime embryonic development to term. The contrastingly high implantation rate (57–71%) and low fetal (5–16%) and full-term (2–3%) developmental rates (Table 3) indicate that several regulatory morphogenic factors and checkpoints may be involved in the development of post-implantation embryos/fetuses.

Our results suggest that, contrary to previous opinion[9], mammals can be reproducibly cloned from adult somatic cells. Furthermore, we believe that the success of these experiments in the mouse provides an amenable model with which to evaluate the molecular mechanisms that regulate the reprogramming of somatic cell genomes, genomic imprinting, embryonic genome activation and cell differentiation. □

Methods

The cloning procedure is summarized in Fig. 5.

Isolation of cumulus cells. Female B6D2F1 (C57BL/6 × DBA/2 used in series A and B), B6C3F1 (C57BL/6 × C3H/He used in series C) or B6C3F1 clones produced in series C were induced to superovulate by consecutive injection of eCG and hCG. 13 h after hCG injection, cumulus–oocyte complexes were collected from oviducts and treated in HEPES–CZB medium[19] supplemented with bovine testicular hyaluronidase (0.1% (w/v), 300 U mg^{-1}) to disperse cumulus cells. We selected cumulus cells of modal (>70%) diameter (10–12 μm) for injection. From preliminary experiments, nuclei from cells with smaller or larger diameters (8–9 or 13–15 μm, respectively) seldom supported development of injected eggs beyond the 8-cell stage (data not shown). Following dispersal, cells were transferred to HEPES–CZB containing 10% (w/v) polyvinylpyrrolidone (average M_r, 360,000) and kept at room temperature for up to 3 h before injection.

Isolation of Sertoli cells and neurons. Sertoli cells were isolated from the testes of 6-month-old B6D2F1 males as described[20], except that HEPES–Ham F-12 medium was used. Manipulation of individual Sertoli cells was done by using a large injection pipette (inner diameter ~10 μm). Neuronal cells were isolated from the cerebral cortex of adult B6D2F1 females. Brain tissue was removed with sterile scissors, quickly washed in erythrocyte-lysing buffer and gently hand-homogenized for several seconds in nucleus isolation medium[21] at room temperature. Nuclei (7–8 μm in diameter) harbouring a conspicuous nucleolus were individually collected from the resulting suspension using the injection pipette before delivery into a recipient enucleated oocyte.

Enucleation of metaphase II oocytes and donor cell nucleus injection. B6D2F1 oocytes (obtained 13 h after hCG injection of eCG-primed females) were freed from the cumulus oophorous and held in CZB medium at 37.5 °C under 5% (v/v) CO_2 in air until required. Groups of oocytes (usually 10–15) were transferred into a droplet of HEPES–CZB containing 5 μg ml^{-1} cytochalasin B, which had previously been placed in the operation chamber on the microscope stage. Oocytes undergoing microsurgery were held with a holding pipette and the zona pellucida 'cored' following the application of several piezo-pulses to an enucleation pipette. The metaphase II chromosome–spindle complex (identifiable as a translucent region) was aspirated into the pipette with a minimal volume of oocyte cytoplasm[22]. After enucleation of all oocytes in one group (~10 min), they were transferred into cytochalasin B-free CZB and held there for up to 2 h at 37.5 °C, then returned to the microscope stage immediately before further manipulation. Nuclei were removed from their respective somatic cells and gently aspirated in and out of the injection pipette (~7 μm inner diameter) until their nuclei were largely devoid of visible cytoplasmic material. Each nucleus was injected into a separate enucleated oocyte within 5 min of its isolation as described[12].

Oocyte activation. Following somatic-cell nucleus injection, some groups of oocytes were placed immediately in Ca^{2+}-free CZB containing both 10 mM Sr^{2+} and 5 μg ml^{-1} cytochalasin B for 6 h. Additional groups of enucleated oocytes injected with cumulus cell nuclei were left in CZB medium at 37.5 °C under 5% (v/v) CO_2 in air for 1–6 h before activation by Sr^{2+} in the presence of 5 μg ml^{-1} cytochalasin B. Sr^{2+} treatment activated the oocytes[23], whereas cytochalasin B prevented subsequent polar-body formation and therefore chromosome expulsion. Following activation, all resulting embryos were transferred to Sr^{2+}-free, cytochalasin B-free CZB medium and incubation was continued at 37.5 °C under 5% (v/v) CO_2 in air.

Embryo transfer. Where appropriate, 2- to 8-cell embryos or morulae/blastocysts were respectively transferred into oviducts or uteri of foster mothers (CD-1, albino) that had been mated with vasectomized CD-1 males 1 or 3 days previously. Following caesarean section of recipient females at 18.5–19.5 d.p.c., live young were raised by lactating CD-1 foster mothers.

DNA typing. DNA from the following control strains and hybrids was obtained from spleen tissue: C57BL/6J (B6), C3H/HeJ (C3), DBA/2J (D2), B6C3F1 and B6D2F1. DNA from the three cumulus cell donor females (B6C3F1), the three oocyte recipient females (B6D2F1) and the three foster females (CD-1) was prepared from tail-tip biopsies. DNA from the six B6C3F1-derived, cloned offspring was prepared from their associated placentas. For the microsatellite markers *D1Mit46*, *D2Mit102* and *D3Mit49*, primer pairs (MapPairs) were purchased from Research Genetics and typed as described[24], except that PCR was carried out for 30 cycles and products were separated by 3% agarose gels (Metaphor) and visualized by ethidium bromide staining. Endogenous ecotropic murine leukaemia provirus DNA sequences (*Emv* loci) were identified following hybridization of *Pvu*II-digested genomic DNA to the diagnostic probe, pEc-B4 (ref. 25). Probe labelling, Southern blotting and hybridization procedures have been described[26].

Received 23 December 1997; accepted 19 June 1998.

1. Campbell, K. H. S., Loi, P., Otaegui, P. J. & Wilmut, I. Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1**, 40–45 (1996).
2. Kono, T. Nuclear transfer and reprogramming. *Rev. Reprod.* **2**, 74–80 (1997).
3. Campbell, K. H. S., McWhir, J., Ritchie, W. A. & Wilmut, I. Sheep doned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–66 (1996).
4. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. S. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813 (1997).
5. Schuetz, A. W., Whittingham, D. G. & Snowden, R. Alterations in the cell cycle of mouse cumulus granulosa cells during expansion and mucification *in vivo* and *in vitro*. *Reprod. Fertil. Dev.* **8**, 935–943 (1996).
6. Wassarman, P. The biology and chemistry of fertilization. *Science* **235**, 553–560 (1987).
7. Epstein, C. J. *The Consequences of Chromosome Imbalance* (Cambridge University Press, 1986).
8. Yanagida, K., Yanagimachi, R., Perreault, S. D. & Kleinfeld, R. G. Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. *Biol. Reprod.* **44**, 440–447 (1991).
9. McGrath, J. & Solter, D. Inability of mouse blastmere nuclei transferred to enucleated zygotes to support development *in vitro*. *Science* **226**, 1317–1319 (1984).
10. Willadsen, S. M. Nuclear transplantation in sheep embryos. *Nature* **320**, 63–65 (1986).
11. Collas, P. & Barnes, F. L. Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. *Mol. Reprod. Dev.* **38**, 264–267 (1994).
12. Czolowska, R., Modlinski, J. A. & Tarkowski, A. K. Behavior of thymocyte nuclei in non-activated and activated mouse oocytes. *J. Cell Sci.* **69**, 19–34 (1984).
13. Tsunoda, T. & Kato, Y. Nuclear transplantation of embryonic stem cells in mice. *J. Reprod. Fertil.* **98**, 537–540 (1993).
14. Tsunoda, T., Tokunaga, T., Imai, I. & Uchida, T. Nuclear transplantation of male primordial germ cells in the mouse. *Development* **107**, 407–411 (1989).
15. Kono, T., Ogawa, M. & Nakahara, T. Thymocyte transfer to enucleated oocytes in the mouse. *J. Reprod. Dev.* **39**, 301–307 (1993).
16. Kimura, Y. & Yanagimachi, R. Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**, 709–720 (1995).

17. Kimura, Y. & Yanagimachi, R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121, 2397–2405 (1995).
18. Eppig, J., Wigglesworth, K., Pendola, F. & Hirao, Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol. Reprod.* 56, 976–984 (1997).
19. Chatot, C. L., Lewis, J. L., Torres, I. & Ziomek, C. A. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol. Reprod.* 42, 432–440 (1990).
20. Erickson, R. P., Zwingman, T. & Ao, A. Gene expression, X-inactivation, and methylation during spermatogenesis: the case of *Zfx*, *Zfa* and *Zfy* in mice. *Mol. Reprod. Dev.* 35, 114–120 (1993).
21. Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* 55, 789–795 (1996).
22. Kono, T., Sotomaru, Y., Sato, Y. & Nakahara, T. Development of androgenetic mouse embryos produced by *in vitro* fertilization of enucleated oocytes. *Mol. Reprod. Dev.* 34, 43–46 (1993).
23. Ben-Mlllich, A., Whittingham, D. G. & Kones, K. T. Meiotic and Mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* 182, 172–179 (1997).
24. Dietrich, W. et al. A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics* 131, 423–447 (1992).
25. Taylor, B. A. & Rowe, L. A mouse linkage testing stock possessing multiple copies of the endogenous ecotropic murine leukemia virus genome. *Genomics* 5, 221–232 (1989).
26. Johnson, K. R., Cook, S. A. & Davisson, M. T. Chromosomal localization of the murine gene and two related sequences encoding high-mobility-group I and Y proteins. *Genomics* 12, 503–509 (1992).

Acknowledgements. This study was supported in part by grants from the National Institutes of Health, ProBio America Inc., and fellowships from the Japanese Society for the Promotion of Science (T.W.) and European Molecular Biology Organization (A.C.F.P.). We thank H. Tateno for chromosome analysis, Y. Nakamura for help with DNA fingerprinting, H. Kishikawa, T. Kasai and R. Kleinfeld for assistance in preparing this manuscript, and J. Eppig for help and advice.

Correspondence and requests for materials should be addressed to R.Y.

Defects in somite formation in *lunatic fringe*-deficient mice

Nian Zhang & Thomas Gridley

The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609-1500, USA

Segmentation in vertebrates first arises when the unsegmented paraxial mesoderm subdivides to form paired epithelial spheres called somites[1,2]. The Notch signalling pathway is important in regulating the formation and anterior–posterior patterning of the vertebrate somite[3–7]. One component of the Notch signalling pathway in *Drosophila* is the *fringe* gene, which encodes a secreted signalling molecule required for activation of Notch during specification of the wing margin[8–11]. Here we show that mice homozygous for a targeted mutation of the *lunatic fringe* (*Lfng*) gene, one of the mouse homologues[12,13] of *fringe*, have defects in somite formation and anterior–posterior patterning of the somites. Somites in the mutant embryos are irregular in size and shape, and their anterior–posterior patterning is disturbed. Marker analysis revealed that in the presomitic mesoderm of the mutant embryos, sharply demarcated domains of expression of several components of the Notch signalling pathway are replaced by even gradients of gene expression. These results indicate that *Lfng* encodes an essential component of the Notch signalling pathway during somitogenesis in mice.

The *Lfng* gene is expressed during somitogenesis in mice in a dynamic pattern that suggests a possible role for *Lfng* in regulating somite formation and establishing somite borders[12,13]. To analyse the role of the *Lfng* gene during embryogenesis, we constructed a targeting vector that deleted 0.7 kilobases (kb) of genomic sequence encoding the putative signal peptide and proprotein region of the Lfng protein, and replaced the deleted sequence with the *lacZ* gene of *Escherichia coli* (Fig. 1a, b). Mice heterozygous for the $Lfng^{LacZ}$ mutant allele were viable and fertile. The pattern of RNA expression from the $Lfng^{LacZ}$ mutant allele was identical to that of *Lfng* RNA expression, but expression of β-galactosidase protein from the $Lfng^{LacZ}$ mutant allele was not useful as a marker for visualizing the normal pattern of *Lfng* expression during somitogenesis owing to the perdurance of the β-galactosidase protein and the dynamic nature of the *Lfng* expression pattern (Fig. 1c–e).

At birth, $Lfng^{LacZ}$ homozygous neonates had a shortened trunk with a rudimentary tail (Fig. 2a). Some $Lfng^{LacZ}$ homozygous neonates died within a few hours of birth, apparently from respiratory difficulties due to malformed rib cages (see below), but other, less severely affected, $Lfng^{LacZ}$ homozygotes could survive to adulthood. Analysis of stained skeletal preparations revealed substantial defects in formation of the vertebral column and ribs in the $Lfng^{LacZ}$ homozygotes (Fig. 2b, c). The regular metameric pattern of the vertebrae was disrupted along the entire longitudinal axis. The ribs of the $Lfng^{LacZ}$ homozygotes were bifurcated and fused, and some ribs were detached from the vertebral column (Fig. 2c).

$Lfng^{LacZ}$ homozygous mutant embryos could be distinguished

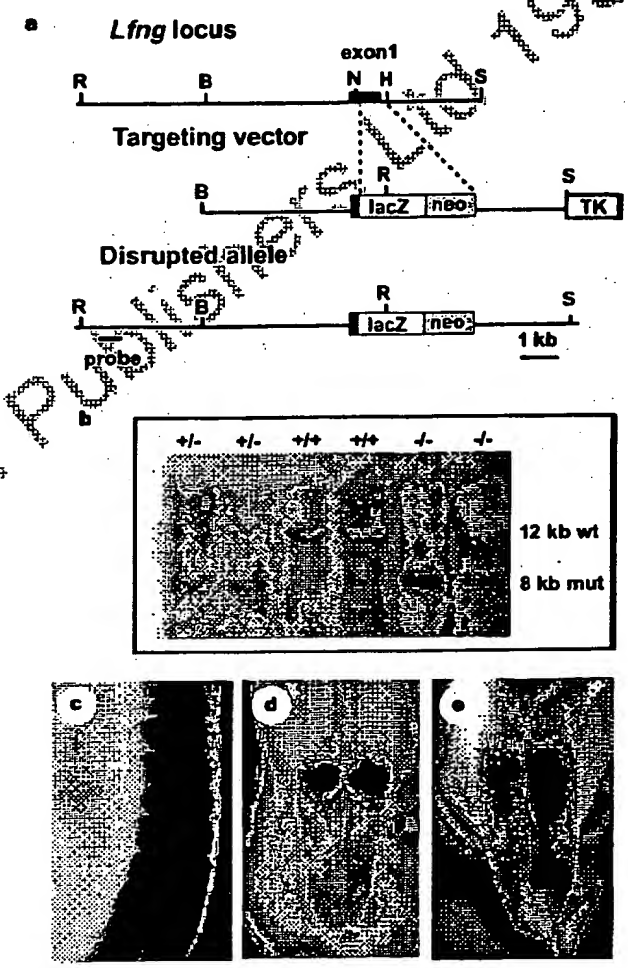


Figure 1 Targeted disruption of the *Lfng* gene. **a**, Targeting scheme. The top line shows the genomic organization of a portion of the *Lfng* gene; the middle line shows the structure of the targeting vector. A 0.7-kb deletion was created which removes most of exon 1, replacing it with the *lacZ* gene and a *neo* cassette. At the bottom is the predicted structure of the *Lfng* locus following homologous recombination of the targeting vector. The probe used for Southern blot analysis in **b** is indicated. Restriction enzymes: B, *Bam*HI; H, *Hin*dIII; N, *Not*I; R, *Eco*RV; S, *Sal*I. **b**, DNA isolated from embryos of the intercross of $Lfng^{LacZ}$/+ heterozygous mice was digested with *Eco*RV, blotted, and hybridized with the indicated probe. Genotypes of progeny are indicated at the top of each lane. **c**, β-Galactosidase expression in $Lfng^{LacZ}$/+ heterozygous embryos revealed that, unlike *Lfng* RNA, β-galactosidase protein was expressed throughout the rostral presomitic mesoderm and the most recently formed somites. **d**, *In situ* hybridization of a $Lfng^{LacZ}$/+ heterozygous embryo with a *lacZ* probe revealed the same banding pattern in the presomitic mesoderm as is observed with a *Lfng* probe, demonstrating that the constitutive expression observed in **c** is due to perdurance of β-galactosidase. **e**, *In situ* hybridization of a $Lfng^{LacZ}$ homozygous mutant embryo with a *lacZ* probe revealed that the stripe of *lacZ* RNA expression is expanded and is more diffuse than in $Lfng^{LacZ}$/+ heterozygous embryos. In **c**–**e**, posterior is at the bottom. **c**, Sagittal view; **d**, **e**, dorsal views.

Cloned rabbits produced by nuclear transfer from adult somatic cells

Patrick Chesné[1], Pierre G. Adenot[1], Céline Viglietta[1], Michel Baratte[2], Laurent Boulanger[1], and Jean-Paul Renard[1*]

We have developed a method to produce live somatic clones in the rabbit, one of the mammalian species considered up to now as difficult to clone. To do so, we have modified current cloning protocols proven successful in other species by taking into account both the rapid kinetics of the cell cycle of rabbit embryos and the narrow window of time for their implantation after transfer into foster recipients. Although our method still has a low level of efficiency, it has produced several clones now proven to be fertile. Our work indicates that cloning can probably be carried out successfully in any mammalian species by taking into account physiological features of their oocytes and embryos. Our results will contribute to extending the use of rabbit models for biomedical research.

The rabbit is gaining attention in biotechnology because it offers several advantages over other laboratory animals for the study of several human physiological disorders[1,2]. Not only can physiological manipulations in this species be more easily carried out than in mice because of its larger size, but it is also phylogenetically closer to primates than are rodents[3]. Currently, the use of rabbits is limited to large-scale production of foreign proteins[4]. Thus cloning, associated with the genetic modifications of donor cells, would greatly enhance the possible use of this species in biotechnology. In contrast to several other mammalian species, however, the rabbit has not been very amenable to somatic cloning[5,6], despite its pioneer role in defining nuclear transfer (NT) methods in mammals[7]. Here we describe a method that has allowed us to produce several healthy and fertile somatic clones of rabbit at about the same frequency as other mammalian species. Our results will contribute to extending the use of rabbit models for biotechnological applications.

Results and discussion

Rabbit NT embryos were reconstructed by electrofusion of freshly collected cumulus cells with recipient enucleated metaphase II (MII) ooplasm. We chose this type of nuclear donor cells because they had been initially used as models to demonstrate the feasibility of somatic cloning[8,9]. Confocal observations of reconstructed embryos, fixed 1 h after electrofusion, showed that donor nuclei exposed to MII ooplasm had condensed into chromosomes[10] (Fig. 1A). Instead of orderly chromosome arrays typical of MII oocytes, we observed misaligned metaphase plates, very similar to those previously shown in the mouse to be compatible with full-term development[8]. We therefore activated NT embryos through a second set of electrostimulation and incubated them in the presence of cycloheximide (CHX; a protein synthesis inhibitor) and 6-dimethylaminopurine (6-DMAP; a kinase inhibitor), two drugs known to facilitate the exit from artificially activated MII stage, but with potential detrimental side effects on the cell cycle[11,12]. Because the rabbit zygote enters S phase very early after activation[13], we focused on reducing the time of exposure to these inhibitors. We observed that they accelerated pronuclear formation in artificially activated rabbit oocytes, but also caused high rates of parthenogenetic development to the blastocyst stage (90%, $n = 130$), even when the time of incubation was reduced to 1 h. Upon removal of the inhibitors, 72% ($n = 25$) of NT embryos exhibited interphasic structures (Fig. 1B), and 1 h later all were in interphase (Fig. 1C). When left in culture, 47% ($n = 135$) developed into blastocysts at day 3 (D3). Their growth as determined from cell number counts at D3 and D4 was slower than that of blastocysts derived from *in vivo* or *in vitro* zygotes. At D4, the number of cells in NT blastocysts was similar to that of *in vivo* or *in vitro* zygotes at D3 (Fig. 2A).

In rabbits, a rapid and significant expansion of blastocysts occurs *in vivo* and stretches the surrounding walls of the uterus so that their individual positions on the uterine horns become easily recognizable as "implantation sites" as early as D6 (ref. 14). Implantation, however, starts only at D7.5, with the progressive dissolution of blastocyst coverings apposed during the transit of the embryo through the female genital tract, thereby contributing to the narrow window of implantation in this species. Upon dissection of the uterine horns at D8, we found that NT embryos could form some implantation sites (7 out of the 91 embryos transferred, 7.7%) following their transfer into synchronous recipients mated with a vasectomized male at the same time as donor females were mated. However, no embryonic structures were seen. Transfer into asynchronous recipients mated 16 h after the donor females were mated resulted in an increase in the implantation rate, which became only slightly lower than controls (12/59 or 20.3%, and 15/54 or 27.8%, respectively). Under these conditions we could recover embryos at the advanced blastocyst stage (see photo in Fig. 2B), but still surrounded by thin coverings of extracellular material, and thus, equivalent to D7 normal embryos[14]. None of the recipients transplanted either synchronously or asynchronously (–16 h) could be diagnosed pregnant at midgestation (Table 1), even when co-transferred with unmanipu-

[1]*Unité de Biologie du Développement et Biotechnologie, Institut National de la Recherche Agronomique, F-78352 Jouy-en-Josas, France.* [2]*Unité Commune d'Expérimentation Animale, Institut National de la Recherche Agronomique, F-78352 Jouy-en-Josas, France.* **Corresponding author: (renard@jouy.inra.fr).*

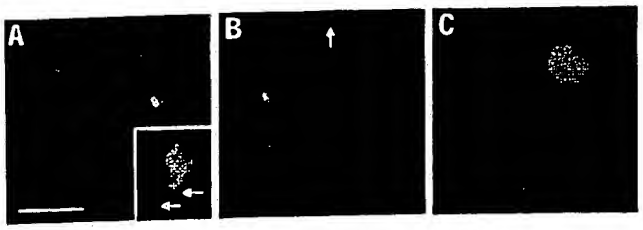


Figure 1. Confocal images of unicellular NT embryos immunolabeled with the anti-α-tubulin antibody (green) and DNA stained with propidium iodide (red). (A) Before the second set of electrostimulation, a misaligned metaphase plate was associated with the spindle, and sometimes individual chromosomes (arrows in the insert) were localized near the spindle poles (insert: 3-fold magnified view of the spindle region). (B) Upon removal of CHX and 6-DMAP, 72% of NT embryos (n = 25) showed a small nucleus and an interphasic microtubular network (arrow). (C) One hour after removal of the drugs, all NT embryos were in interphase and 71% (n = 17) exhibited a single and large pronucleus-like nucleus like those observed in normal rabbit zygotes (not shown). Bar, 50 μm.

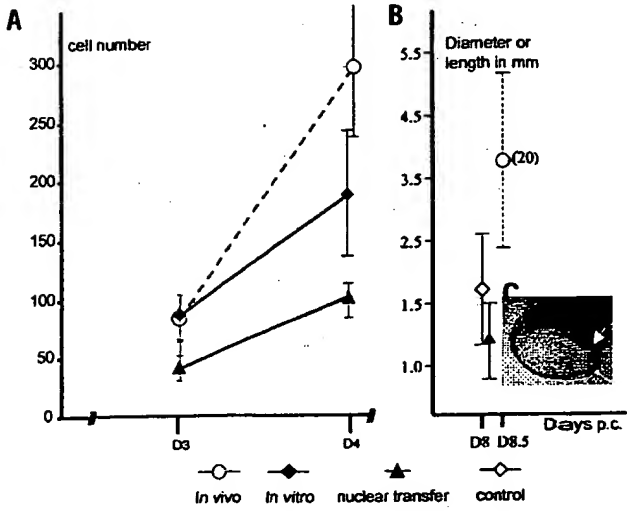


Figure 2. Development of rabbit blastocysts reconstructed with cumulus cells or derived from *in vivo*–fertilized embryos. (A) *In vitro* increase in cell number (mean ± s.e.) between D3 and D4 of embryos either recovered directly from donors (*in vivo*, n = 27), or cultured from the one-cell stage (~20 h post-hCG) after either natural mating (*in vitro*, n = 44) or nuclear transfer (NT, n = 31). (B) Mean diameters or lengths (in mm ; ± s.e.) of the embryonic disks of D8 blastocysts recovered directly from donors (*in vivo*)[20], following transfer into recipients at the one-cell stage after natural mating (control, n = 9), or resulting from NT (n = 7). (C) Example of a retarded NT blastocyst recovered at D8 after transfer at the four-cell stage into a −16 h asynchronous recipient; embryonic disk (large arrow) is visible but the blastocyst is still surrounded by a thin layer of embryo covering (small arrow) that should normally have disappeared at D7 (ref. 14).

lated "helper" embryos of another strain (Fauve-de-Bourgogne), or transferred with an excess of NT embryos (up to 39 per female; data not shown). These observations suggested that only very few NT blastocysts could implant because their development was too delayed. In one case, we could observe a D8 NT blastocyst already adhering to the uterine epithelium and very similar in size to normal implantation controls. We therefore extended the asynchrony between donor and recipient females from 16 to 22 h. Such a marked asynchrony at early cleavage stages of development had not been attempted previously with NT embryos, but can be compatible with full-term development of fertilized eggs[15,16]. Under these conditions, 10 out of 27 (37%) of the −22 h asynchronized recipients were diagnosed pregnant after palpation at D14. Four of these gave birth at D31 to six live kits (Fig. 3) weighing 30–90 g (mean value, 65 g). Such variability is also observed with kits born from reduced litter sizes (one to four fetuses) occasionally obtained in our facilities. Expression of a green fluorescent protein (GFP) transgenic marker from hair follicles (Fig. 3) and from lymphocytes (not shown) confirmed that kits resulted from NT of cumulus cells. Two kits of normal morphological appearance (respective weights 90 and 30 g) died one day after birth, for one of whom we suspect failure in the adoptive process from the lactating mother. The four others are developing normally, and two of them (Fig. 3, B1), when tested for fertility by natural mating, gave birth to seven and eight healthy kits, respectively.

In conclusion, our results show that the former limitations to successful rabbit somatic cloning have been overcome by taking into account species differences in oocyte physiology and early embryonic development. Both a shortened timing for otherwise classical activation procedures and the transfer of reconstructed embryos into recipients retarded by nearly one day had a decisive influence on the *in vivo* development of NT embryos. The maximization of the developmental response of rabbit oocytes to external activating stimuli, through controlled Ca^{2+} stimulation regimes[17] and characterization of the embryonic signals that regulate rabbit uterine epithelial responsiveness at implantation[18], should help to improve term survival rates of embryos reconstructed with different types of somatic and cultured cells.

Table 1. *In vivo* development of rabbit somatic nuclear transfer embryos

Type of recipients	Synchronous	Asynchronous (−16 h)	Asynchronous (−22 h)
Stage of embryos	1-cell	1-cell	4-cell
No. of reconstructed embryos	554	523	775
[No. of replicates]	[19]	[18]	[27]
No. of fused embryos	427	346	612
[% from reconstructed]	[77.1]	[66.2]	[79.0]
Total transferred	367	346	371
[% from fused]	[100.0]	[100.0]	[60.6]
No. of recipients transferred	19	18	27
No. of recipients pregnant at day 14	0	0	10
[% from transferred]			[37.0]
No. of recipients delivering	0	0	4[a]
[% from transferred]			[14.8]
No. of kits born			6
[% from embryos transferred]			[1.6]
Alive at weaning			4
Mean weight of kits at birth (g)			65 ± 20[b]

[a]Abortions between day 15 and day 29 of pregnancy (13 cotyledons and degenerated fetuses recovered).
[b]Mean weight of kits at birth in our facilities: 55.8 ± 17.0 g (sample size, n = 51).

Experimental protocol

Source of oocytes and cumulus cells. MII oocytes were collected from superovulated does of New-Zealand breed 16 h after human chorionic gonadotrophin (hCG) injection and mating to a vasectomized male. They were incubated in 0.5% hyaluronidase (catalog no. H3506; Sigma, St. Louis, MO) for 15 min to remove cumulus cells by gentle pipetting. For nuclear transfer, oocytes were enucleated as described[19]. All manipulations were done in M199 (Life Technologies, Rockville, MD), 20 mM HEPES (Sigma), supplemented with 10% vol/vol FCS (Life Technologies).

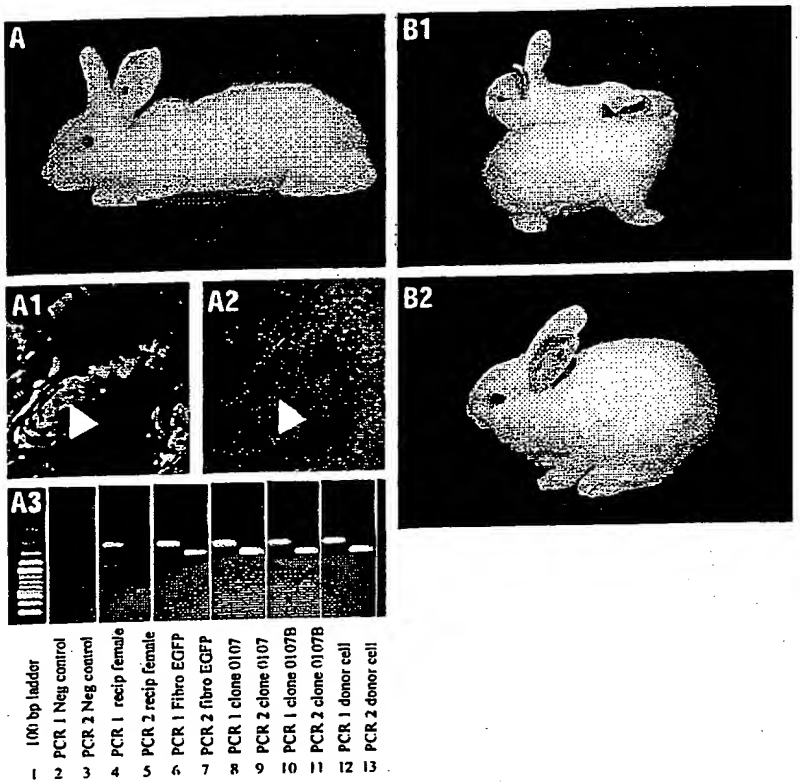


Figure 3. Rabbits born from somatic nuclear transfer. (A) Cloned rabbit 0107 with corresponding controls: (A1) expression of the EGFP protein fluorescence (arrowhead) detected by confocal microscopy from hair follicles obtained from an ear biopsy at 1 month of age; (A2) the same under transmission light; (A3) amplifications of the *EGFP* transgene (PCR 2) and of the exon 10 of the *CFTR* gene used as DNA quality control (PCR 1) with expected fragment sizes of 240 bp for the *CFTR* gene and 350 bp for the *EGFP* transgene. This confirms that rabbit 0107 and its littermate 107b (who died 1 day after birth) were derived from the donor cumulus cell. (B1, B2) Three other rabbits from two different litters; rabbits in B1 have now proved to be fertile.

Contemporary cumulus cells were obtained from the New Zealand breed or F1 New Zealand × Fauve-de-Bourgogne or F1 transgenic New Zealand females harboring a DNA construct with the coding sequence of the enhanced green fluorescent protein (EGFP) placed under the control of an elongation factor 1 (EF1) promoter. EGFP fluorescence and PCR amplification were used as markers of donor cumulus cells. These were kept at 38°C in Ca^{2+},Mg^{2+}-free PBS supplemented with 1% polyvinylpyrrolidone (PVP) 40,000 (Sigma) before being used as a source of nuclei.

Oocyte activation and nuclear transfer. To reconstruct NT embryos, individual cumulus cells were inserted by micromanipulation under the zona pellucida of the enucleated oocytes. NT embryos and MII oocytes were activated 18–20 h post-hCG as follows. Two sets of electrical stimulation were applied 1 h apart with a BTX stimulator (Biotechnologies & Experimental Research Inc., San Diego, CA) (3 DC pulses of 3.2 kV/cm for 20 µs each in mannitol 0.3 M in water containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$). The first set induced the cumulus cell–oocyte fusion. Reconstructed em-bryos and oocytes were then incubated for 1 h in M199 at 38°C. Then, the second set of pulses was applied to induce activation. NT embryos and oocytes were incubated for 1 h at 38°C in M199 containing 5 µg/ml CHX (Sigma) and 2 mM 6-DMAP (Sigma), then ret-urned to culture in a 50 µl microdrop of B2 medium (Laboratoire CCD, Paris, France) supplemented with 2.5% FCS under mineral oil (catalog no. M8410; Sigma) at 38°C under 5% CO_2 in air.

Analysis of preimplantation stages. Microtubule organization and chromatin in one-cell NT embryos were observed as already described[19], except that fixation lasted 20 min at 37°C and the mounting medium was Vectashield (Vector Laboratories, Burlingame, CA). Development rates until blastocyst stage were assessed after *in vitro* culture for 3 and 4 days. For cell number evaluation, embryos were fixed as above, stained with Hoechst 33342 at a concentration of 1 µg/ml, then mounted on well slides in Vectashield and monitored under epifluorescence.

Analysis of peri-implantation stages and *in vivo* development. Recipient females were mated to vasectomized males either at the same time (synchronous recipients), or 16 h or 22 h after the oocyte donors (asynchronous recipients). NT embryos were transplanted surgically through the infundibulum into each oviduct of recipients either at the one-cell stage (1–3 h post-activation) or at the four-cell stage (after an overnight culture). Implantation rate was assessed after killing of recipients at day 8 (D8). When visible, embryonic disks of blastocysts were measured microscopically (160×). Pregnancy was determined by palpation 13 or 14 days after embryo transplantation and the pregnant recipients delivered by caesarian section at 31 days post mating.

PCR analysis. The presence of the GFP transgenic marker was detected by PCR using a sense (5′-GAGTTTG-GATCTTGGTTCAT-3′) and an antisense (5′-GGCACGGGCAGCTTGCCGGTGG-3′) primer (Genset, Paris, France). To control the DNA quality, PCR was performed on 300–400 ng of DNA prepared with tissue extraction kit (Qiagen, Valencia, CA) with the sense primer, 5′-TTTTCCTGGATCATGCCTGGCAC-3′, and the antisense primer, 5′-CTACCTGTAGCAGCTTACCCA-3′, covering the exon 10 of the rabbit *CFTR* gene (Genset). Negative controls were double-distilled water and recipient female DNA, while positive controls were DNA from transgenic cultured fibroblasts.

Acknowledgments

We thank C. Thibault for his constant support to our work, C. Viebahn for stimulating discussions, the staff from our rabbit facility for their critical role in the care of recipients and cloned kits, C. Poirier for assistance in embryo transfer, E. Campion for the recovery and fixation of D8 embryos, and C. Young and B. Nicolas for help during the preparation of the manuscript. This work was supported by a grant from the Association Française de Lutte contre la Mucoviscidose (AFLM).

Competing interests statement

The authors declare that they have no competing financial interests.

Received 18 October 2001; accepted 23 January 2002

1. Hoeg, J.M. *et al.* Overexpression of lecithin: cholesterol acyltransferase in transgenic rabbits prevents diet-induced atherosclerosis. *Proc. Natl. Acad. Sci. USA* 93, 11448–11453 (1996).
2. Chen, J.M. *et al.* A combined analysis of the cystic fibrosis transmembrane conductance regulator: implications for structure and disease models. *Mol. Biol. Evol.* 18, 1771–1788 (2001).
3. Graur, D., Duret, L. & Gouy, M. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature* 379, 333–335 (1996).
4. Stinnackre, M.G., Massoud, M., Viglietta, C. & Houdebine, L.M. The preparation of recombinant proteins from mouse and rabbit milk for biomedical and pharmaceutical studies. In *Trangenic animals, generation and use;* (ed. Houdebine, L.M.) 461–463 (Harwood Academic Publishers, Amsterdam 1997).
5. Yin, X.J., Tani, T., Kato, Y. & Tsunoda, Y. Development of rabbit parthenogenetic oocytes and nuclear-transferred oocytes receiving cultured cumulus cells. *Theriogenology* 54, 1460–1476 (2000).
6. Dinnyés, A. *et al.* Development of cloned embryos from adult rabbit fibroblasts: effect of activation treatment and donor cell preparation. *Biol. Reprod.* 64, 257–263 (2001).

7. Bromhall, J.D. Nuclear transplantation in the rabbit egg. *Nature* **258**, 719–722 (1975).
8. Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. & Yanagimachi, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–374 (1998).
9. Wells, D.N., Misica, P.M. & Tervit, H.R. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60**, 996–1005 (1999).
10. Campbell, K.H.S., Loi, P., Otaegui, P.J. & Wilmut, I. Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1**, 40–46 (1996).
11. Soloy, E. *et al.* Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. *Biol. Reprod.* **57**, 27–35 (1997).
12. Meyer, L. & Kim, S.H. Chemical inhibitors of cyclin-dependent kinases. *Methods Enzymol.* **283**, 113–128 (1997).
13. Szöllösi, D. Time and duration of DNA synthesis in rabbit eggs after sperm penetration. *Anat. Rec.* **154**, 209–212 (1966).
14. Denker, H.W. Implantation: the role of proteinases and blockage of implantation by proteinase inhibitors. *Adv. Anat. Embryol. Cell Biol.* **53**, 1–123 (1981).
15. Heape, W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Proc. R. Soc. Lond. A/B* **48**, 457–458 (1890).
16. Chang, M.C. Development and fate of transferred rabbit ova or blastocysts in relation to the ovulation time of recipients. *J. Exp. Zool.* **114**, 197–226 (1950).
17. Ozil, J.P. & Huneau, D. Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. *Development* **128**, 917–928 (2001).
18. Hoffman, L.H., Olson G.E., Carson, D.D. & Chilton, B.S. Progesterone and implanting blastocysts regulate MUC1 expression in rabbit uterine epithelium. *Endocrinology* **139**, 266–271 (1998).
19. Adenot, P.G., Szöllösi, M.S., Chesné, P., Chastant, S. & Renard, J.P. *In vivo* aging of oocytes influences the behavior of nuclei transferred to enucleated rabbit oocytes. *Mol. Reprod. Dev.* **46**, 325–336 (1997).
20. Gottschewski, G.H.M. & Zimmermann, W. *Die Embryonalentwicklung des Hauskaninchens. Normogenese und Teratogenese.* Ch. 3.3,103–117 (M. & H. Schaper, Hannover; 1973).

V. LANDA

Institute of Animal Physiology and Genetics, Czechoslovak Academy of Sciences, 277 21 Liběchov

Factors Influencing the Results of Transfers of Rabbit Embryos Stored at −196° C

(dimethyl sulphoxide / culture / transfer to the recipients / implantation)

Received January 9, 1981

Abstract. Rabbit embryos at the 8-cell and morula stages were frozen and stored at −196°C for 2−200 days. After thawing the embryos were examined for their viability in vitro and in vivo. In vitro, 62.5% of frozen 8-cell embryos and 81.4% of frozen morulae developed to blastocysts. In the control group of unfrozen embryos, 93.2% 8-cell embryos and 92.4% morulae developed to the blastocyst stage. Culture permitted a more reliable elimination of the embryos damaged during freezing and thawing. Embryos were transferred into the reproductive tracts of the recipients either directly after thawing or after 24 h in culture. Synchronous transfers of frozen rabbit embryos were not successful. After asynchronous transfers of morulae and blastocysts into the oviducts, implantation was 31.8% and 42.9%, respectively. After transfer of blastocysts into the uterine horns of the recipients, 47.6% embryos implanted.

Whittingham, Leibo and Mazur (1972) were the first to obtain viable young from embryos stored at −196°C upon transfer to foster mothers. With minor modifications, the method for mouse embryo storage has been used to store other mammalian embryos (Wilmut and Rowson 1973, Bank and Maurer 1974, Willadsen et al. 1974, Whittingham 1975, Bilton and Moore 1976). Only in mice were the results with embryos stored at −196°C comparable to those obtained with unfrozen embryos (Whittingham et al. 1977). In other mammalian species, the survival of frozen embryos is poor when compared with that of unfrozen embryos, particularly after transfer to foster mothers. Viability of frozen embryos after transfer is very low in rabbits. Only 10−15% of the embryos showed normal development after transfer (Bank and Maurer 1974, Whittingham and Adams 1976, Maurer and Haseman 1976). Better results were only exceptionally obtained (Tsunoda and Sugie 1977a). Why rabbit embryos stored at −196°C have low viability remains unknown.

In this study we have examined the factors which may markedly affect the results of transfers and which have not been investigated in detail previously: the selection of embryos for transfer and the effect of synchronization between donor and recipient.

Abbreviations: DMSO — dimethyl sulphoxide, HCG — human chorionic gonadotropin, FSH — follicle stimulating hormone, IU — international units.

MATERIAL AND METHODS

Recovery of embryos

Sexually mature female rabbits of various breeds were induced to superovulate by the subcutaneous injection of follicle stimulating hormone (Folicotropin, Spofa, Praha, Czechoslovakia) as described by Kennelly and Foote (1965). Twenty-four h after the last subcutaneous injection of FSH, the females were naturally mated and at the same time they received an intravenous injection of 100 IU of human chorionic gonadotropin (Preadyn, Spofa, Praha). Eight- to 16-cell embryos were obtained by flushing the oviducts 44 h after mating and HCG injection. Morulae (Plate XXI, Fig. 1/1) were flushed from the oviducts and uterine horns 68 h after mating and HCG. Only embryos of the desired type and with intact zona pellucida and mucin coat were used in further experiments. The medium used for flushing, freezing, culture and transfer was Krebs-Ringer's phosphate-buffered saline (pH 7.4) supplemented with lyophilized growth proteins of calf serum, 5 to 10 mg/ml (Institute of Sera and Vaccines, Praha), 5.5% isotonic glucose, 0.1 ml/ml. streptomycin, 50 μg/ml and penicillin, 50 IU/ml.

Freezing and thawing of embryos

The method of Whittingham and Adams (1976) was modified as follows: After 5 min in medium with 0.5 M dimethyl sulphoxide and a further 5 min in 1.0 M DMSO, the embryos were transferred to vessels containing 0.1 to 0.3 ml of medium with 1.5 M DMSO. Embryos in groups of 10—15 were frozen in test tubes (1.5 × 8 cm) or plastic straws (0.5 ml) which were cut to 6 cm. After 10 min in medium with 1.5 M DMSO, the samples in test tubes and sealed plastic straws were transferred from room temperature directly to a cooling bath at −6°C. Two or three min later, the crystallization of the medium was induced by local cooling the walls of vessels. The rate of cooling was 1.0—1.3°C/min between −6 and −110°C. Freezing was performed in a ultracryostat N 180 (Prüfgeräte-Werk, Medingen, GDR). Upon reaching −110°C, the samples were transferred into liquid nitrogen and stored for 2—200 days.

The samples frozen to −196°C were thawed at rates of 7—9°C/min. Slow thawing was terminated at −5°C. The samples at −5°C were rapidly warmed to room temperature. The DMSO concentration was stepwise diluted by transfer of embryos into medium containing 0.7 M and 0.5 M DMSO, respectively, each time for 3—4 min. Embryos were kept in medium without DMSO for 10—30 min before further used.

The viability of embryos was checked by culture and by transfer into the recipients.

Culture of embryos

Embryos were cultured in drops of culture medium under paraffin oil (Brinster 1963) for 24—48 h at 37.5°C in an atmopshere of 5% CO_2 in air. Modified TCM 199 (Pavlok and McLaren 1972) was used as culture medium. For culture, the embryos after thawing were selected as follows: in experiment A/1 all embryos obtained from test tubes after thawing were used. In experiment A/2 undamaged embryos with an intact mucin coat and zona pellucida obtained from plastic straws were cultured. Unfrozen, normally developing embryos were cultured immediately after flushing from the genital tract.

At the end of the culture, the embryos were evaluated at a magnification of 100 to 300 and classified as "blastocysts", i.e., embryos with blastocoel occupying more than half of the embryo, with well visible embryoblast and without apparent damage to the trophoblast, and "degenerate and abnormal embryos", i.e.. all damaged and abnormally developing embryos.

Transfer of embryos

Ovulation in the recipients was induced by an intravenous injection of 50—100 IU of HCG. After laparotomy in the linea alba, the embryos in a small amount of the medium were transferred to the oviducts or uterine horns of the recipients. The number of embryos transferred into one recipient ranged between 5 and 11. Embryos were transferred either directly after thawing or after 24 h in culture. Some embryos were transferred into the recipients in which the length of the luteal phase of the cycle, as given by HCG administration, was synchronized with the embryo's age — "synchronous transfers". The other embryos were transferred into the recipients at a time when the embryos were 24 h older in comparison with the length of the luteal phase of the recipient's cycle — "asynchronous transfers". Individual experiments were arranged as follows: Synchronous transfers — Experiment B/1, 8-cell embryos (age 44 h) were transferred directly after thawing into the ampulla of the oviducts of the recipients used 44—48 h after HCG. Experiment B/2, 8-cell embryos cultured for 24 h were transferred into the uterine horns of the recipients 68—72 h after HCG. Asynchronous transfer — Experiment B/3, morulae (age 68 h) were transferred directly after thawing into the isthmus of the oviducts of the recipients 44—48 h after HCG. Experiment B/4, morulae cultured for 24 h were transferred into the isthmus of the oviducts of the recipients 68—72 h after HCG. Experiment B/5, morulae cultured for 24 h were transferred into the uterine horns of the recipients 68—72 h after HCG injection. Control was performed 12—14 days after the transfer. After laparotomy in the linea

Table 1. In vitro development of rabbit embryos frozen to −196°C and unfrozen embryos

Experiment	Deveop-mental stage	No. embryos frozen/No. embryos after thawing	No. cul-tured embryos	Length of culture (h)	Stage of embryos after culture	
					degenerate and abnormal	blastocysts (%)*
A/1	8-cell	55/48	48	48	18	30 (62.5)
	morula	66/59	59	24	11	48 (81.4)
A/2	8-cell	60/60	30	48	13	17 (56.7)
	morula	86/86	72	24	19	53 (73.6)
Controls	8-cell	—	117	48	8	109 (93.2)
	morula	—	185	24	14	171 (92.4)

* Percentage was calculated from the number of cultured embryos.

alba, the number of implantations was determined. In 5 recipients a further control was made on day 25 after the transfer. Two recipients were allowed to go to term.

RESULTS

The development in vitro of rabbit embryos stored at −196°C and unfrozen embryos is summarized in Table 1. After thawing from test tubes (Exp. A/1), the proportion of 8-cell embryos and morulae developing to blastocysts was 62.5% and 81.4%. respectively. i.e., 54.5% and 72.7% of the total number of frozen embryos. Embryonic fragments and disintegrated coats indicated that embryos, which were lost during manipulation (12.7% 8-cell embryos and 10.6% morulae), had been damaged during freezing or thawing. Among unfrozen embryos, 93.2% 8-cell embryos and 92.4% morulae continued normal development.

Blastocysts derived from the frozen embryos after culture (Plate XXI, Fig. 1/4, 1/5) were comparable to those obtained from the unfrozen embryos (Plate XXI, Fig. 1/2, 1/3). Some embryos with damaged mucin coat (Plate XXI, Fig. 1/6), damaged investments and without investments continued development. Blastocysts developing from embryos with damaged investments were shedding the zona pellucida without preceding expansion. Some embryos with damaged investments rapidly degenerated.

Thirty (50%) 8-cell embryos and 14 (16%) morulae could not be cultured because of the damage to the zona pellucida and mucin coat (Plate XXII, Fig. 2/1—4), absence of investments (Plate XXII, Fig. 2/5), and damage to or loss of embryos (Plate XXII, Fig. 2/6—9). Of the morphologically "normal"

Table 2. Development of rabbit embryos frozen to −196°C after thawing and after transfer to foster mothers

Experiment	Developmental stage frozen	Developmental stage transferred	Recipient: length of the luteal phase of the cycle (h)	Recipient: site of transfer	No. recipients/No. embryos transferred	No. pregnant recipients/No. implantations (%)
B/1	8-cell	8-cell	48	oviduct**	6/56	—
B/2	8-cell	morula*	72	uterus	6/38	1/1 (2.6)
B/3	morula	morula	48	oviduct***	3/22	3/7 (31.8)
B/4	morula	blastocyst*	72	oviduct***	3/28	3/12 (42.9)
B/5	morula	blastocyst*	72	uterus	10/82	9/39 (47.6)

* Embryos cultured for 24 h.
** Transfer into the ampulla.
*** Transfer into the isthmus.

embryos selected for culture 56.7% 8-cell embryos and 73.6% morulae developed into blastocysts. Among frozen embryos, 28.3% 8-cell embryos and 61.6% morulae developed to the blastocyst stage (Exp. A/2). Among embryos selected in experiment A/2 for culture, 13 (43.8%) 8-cell embryos and 19 (26.4%) morulae showed retardation in development (Plate XXIII, Fig. 3/1, 3/2) did not develop (Plate XXIII, Fig. 3/3), only a few cells underwent division (Plate XXIII, Fig. 3/4) or degenerated (Plate XXIII, Fig. 3/5—9). The seemingly low effectiveness of the method in experiment A/2 was due to the elimination of undamaged embryos which had, however, investments damaged and gave generally poorer results during freezing in plastic ampoules. The methods of freezing and thawing used proved more advantageous for more advanced developmental stages (Exp. A/1, A/2).

Table 2 summarizes the results of transfers of rabbit embryos stored at −196°C. Synchronous transfers (Exp. B/1, B/2) were not successful. Of the 12 recipient animals, only one recipient became pregnant. Of the 94 transferred embryos, only one embryo implanted (Exp. B/2).

Asynchronous transfers (Exp. B/3, B/4) were more successful. After transfer of embryos into the oviducts of 6 recipients, all recipients became pregnant on days 12—14 after transfer. Of the 50 transferred embryos, 19 implantations, 31.8% morulae and 42.9% blastocysts (Exp. B/3, B/4) were observed. The best results were obtained with asynchronous transfers of blastocysts into the uterine horns. Of the 10 recipients, 9 became pregnant and of the 82 transferred embryos, 39 (47.6%) implantations were seen, i.e., 52% embryos developed from blastocysts transferred to pregnant recipients (Exp. 5/5).

Normal development of embryos observed on day 25 after transfer in 5 recipients and live young born to 2 recipients confirmed that embryos stored at −196°C are capable of normal development.

DISCUSSION

In our experiments the viability of rabbit embryos frozen to −196°C and thawed was comparable to the results obtained by Bank and Maurer (1974), Whittingham and Adams (1976), Maurer and Haseman (1976) and Tsunoda and Sugie (1977b). The results probably reflect the present possibilities of the method based on slow freezing to temperatures lower than −60°C and on slow thawing. The lower viability of embryos frozen in plastic straws could have been due to some unfavourable properties of plastic material as was also the case in the experiments of Whittingham et al. (1977).

The development in vivo depends on the selection of embryos. High mortality after transfer of damaged and abnormal embryos (Shea et al. 1976, Bilton and Moore 1977, Polge and Willadsen 1978) and the degeneration of damaged embryos during culture, as observed in our experiments, have demonstrated that such embryos are not convenient for transfer. Selection of rabbit embryos according to the morphological condition need not be reliable. We showed that some of the morphologically "intact" embryos degenerated or developed abnormally already after short-term culture. The short-term culture of embryos after freezing proved to be a convenient method for an additional elimination of embryos which had been apparently damaged at the subcellular level during freezing and thawing and it appeared to be suitable for the selection of embryos capable of normal development. If suitable culture systems are used, short-term culture has no adverse effect on further development of embryos (Adams 1970).

In selecting embryos for transfers we also paid attention to the condition of the non-cellular investments of rabbit embryos, the zona pellucida and the mucin coat. Mechanical damage to the investments increases the risks of damage during further manipulation in vitro and often causes degeneration and loss of embryos. In some cases, the embryos continued development, even in damaged investments, and deaths occurred at a later time as a result of their premature shedding the investments. A marked decrease in implantation already after the reduction of the mucin coat has been reported by Greenwald (1962), the necessity of expansion of the blastocyst for the initiation of implantation by Chang (1950) and the effect of the mucin coat on the formation of the rabbit preimplantation blastocyst by Kane (1975). Thus, they have indirectly demonstrated that embryos with damaged investments must be discarded.

All the factors involved in the damage to the investments of rabbit embryos are not yet known. In our experiments we failed to confirm the results

of Whittingham and Adams (1976), who decreased the incidence of embryos with damaged investments from 29% to merely 9% by prolonging slow freezing from −80°C to −110°C. The effectiveness of long-term preservation of rabbit embryos will be increased if the causes of the damage to the investments are known.

Synchronization between donors and recipients is of primary importance for the results of transfer. We have shown that currently used synchronous transfers (Chang and Pickworth 1969, Rowson et al. 1969) are not suitable for rabbit embryos stored at −196°C. This may be due to the retardation in development caused by the sojourn in unfavourable conditions as observed both in frozen embryos (Whittingham 1977) and in embryos stored at 10°C (Anderson and Foote 1975) or in cultured embryos (Bowman and McLaren 1970). During synchronous transfers so affected embryos are placed in an environment which does not correspond to their developmental capacity. The site of transfer also plays a role. The uterine environment proved not suitable, mainly for the early developmental stages (Chang 1950, Adams 1970). Actually the transfer of morulae into the uterine horns of the synchronized recipients may have caused the failure or a low level of implantation not only in our experiments but also in those of Bank and Maurer (1974), Maurer and Haseman (1976) and Whittingham and Adams (1976). The transfer of later developmental stages into the oviducts may favourably affect further development of the embryos (Tsunoda and Sugie 1977a). The results of transfers were markedly improved by asynchronous transfers. More than 50% of the implanting embryos corresponded to the results obtained with unfrozen embryos (Adams 1962, Binkerd and Anderson 1979). It appeared that rabbit embryos, like mouse embryos (Whittingham and Anderson 1976), required a certain time for the restoration of the metabolism and for the desired level of synchronization which was disturbed by storage at very low temperatures.

The high efficacy of frozen embryo transfers suggests that rabbit embryos might be more widely used in various areas of reproduction biology. Some questions, for example, the variations in the number of embryos implanting in individual recipients (10—80%), cannot be answered unless further studies are undertaken. In this regard, factors other than low temperatures are involved, because similar variations are also observed with unfrozen embryos.

REFERENCES

Adams, C. E.: *Studies on prenatal mortality in the rabbit, Oryctolagus cuniculus: The effect of transferring varying numbers of eggs.* J. Endocr., *24* : 471, 1962.

Adams, C. E.: *The development of rabbit eggs after culture in vitro for 1—4 days.* J. Embryol. Exp. Morph., *23* : 21, 1970.

Anderson, G. B., Foote, R. H.: *Effects of low temperature upon subsequent nucleic acid and protein synthesis of rabbit embryos.* Exp. Cell Res., *90* : 73, 1975.

Bank, H., Maurer, R. R.: *Survival of frozen rabbit embryos.* Exp. Cell Res., *89* : 188, 1974.
Bilton, R. J., Moore, N. W.: *In vitro culture, storage and transfer of goat embryos.* Aust. J. Biol. Sci., *29* : 125, 1976.
Bilton, R. J., Moore, N. W.: *Successful transport of frozen cattle embryos from New Zealand to Australia.* J. Reprod. Fert., *50* : 363, 1977.
Binkerd, P. E., Anderson, G. B.: *Transfer of cultured rabbit embryos.* Gamete Res., *2* : 65,1979.
Bowman, P., McLaren, A.: *Cleavage rate of mouse embryos in vivo and in vitro.* J. Embryol. Exp. Morph., *24* : 203, 1970.
Brinster, R. L., *A method for in vitro cultivation of mouse ova from two-cell to blastocyst.* Exp. Cell Res., *32* : 205, 1963.
Chang, M. C.: *Development and fate of transferred rabbit ova or blastocyst in relation to the ovulation time of recipients.* J. Exp. Zool., *114* : 197, 1950.
Chang, M. C., Pickworth, S.: *Egg transfer in the laboratory animal.* In: *The Mammalian Oviduct.* Hafez, E. S. E., Blandau, R. J. eds.. Chicago and London, 1969 (p. 389).
Greenwald, G. S.: *The role of the mucin layer in development of the rabbit blastocyst.* Anat. Rec., *142* : 407, 1962.
Kane, M. T.: *Inhibition of zona shedding of rabbit blastocysts in culture by the presence of a mucin coat.* J. Reprod. Fert., *44* : 539, 1975.
Kennelly, J. J., Foote, R. H.: *Superovulatory response of pre- and postpubertal rabbits to comercially available gonadotropins.* J. Reprod. Fert., *9* : 177, 1965.
Maurer, R. R., Haseman, J. K.: *Freezing morula stage rabbit embryos.* Biol. Reprod., *14* : 256, 1976.
Pavlok, A., McLaren, A.: *The role of cumulus cells and the zona pellucida in fertilization of mouse eggs in vitro.* J. Reprod. Fert., *29* : 91, 1972.
Polge, C., Willadsen, S. M.: *Freezing eggs and embryos of farm animals.* Cryobiology, *15* : 370, 1978.
Rowson, L. E. A., Moor, R. M., Lawson, R. A. S.: *Fertility following egg transfer in the cow; effect of method, medium and synchronization of oestrus.* J. Reprod. Fert., *18* : 517, 1969.
Shea, B. F., Hines, D. J., Lightfoot, D. E., Ollis, G. W., Olson, S. M.: *The transfer of bovine embryos.* In: *Egg Transfer in Cattle.* Rowson, L. E. A. ed. Commission of the European Communities, EUR 5491. Luxembourg 1976 (p. 145).
Tsunoda, Y., Sugie, T.: *Survival of rabbit eggs preserved in plastic straws in liquid nitrogen.* J. Reprod. Fert., *49* : 173, 1977a.
Tsunoda, Y., Sugie, T.: *Effect of the freezing medium on the survival of rabbit eggs after deep freezing.* J. Reprod. Fert., *50* : 123, 1977b.
Whittingham, D. G.: *Survival of rat embryos after freezing and thawing.* J. Reprod. Fert., *43* : 575, 1975.
Whittingham, D. G.: *Some factors affecting embryo storage in laboratory animals.* In: *The Freezing of Mammalian Embryos.* Ciba Foundation Symposium 52 (new series). Elliott, K., Whelan, J. eds., Amsterdam 1977 (p. 97).
Whittingham, D. G., Adams, C. E.: *Survival of rabbit embryos after freezing and thawing.* J. Reprod. Fert., *47* : 269, 1976.
Whittingham, D. G., Anderson, E.: *Ultrastructural studies of frozen-thawed 8-cell mouse embryos.* J. Reprod. Fert., *48* : 137, 1976.
Whittingham, D. G., Leibo, S. P., Mazur, P.: *Survival of mouse embryos frozen to −196°C and −269°C.* Science, *178* : 411, 1972.
Whittingham, D. G., Lyon, M. F., Glenister, P. H.: *Re-establishment of breeding stocks of mutant and inbred strains of mice from embryos stored at −196°C for prolonged periods.* Genet. Res., Camb., *30* : 287, 1977.
Willadsen, S. M., Polge, C., Rowson, L. E. A., Moor, R. M.: *Preservation of sheep embryos in liquid nitrogen.* Cryobiology, *11* : 560, 1974. (Abstr.)
Wilmut, I., Rowson, L. E. A.: *Experiments on the low-temperature preservation of cow embryos.* Vet. Rec., *92* : 686, 1973.

В. Ланда

Факторы, влияющие на результаты пересадки кроличьих эмбрионов, хранившихся при —196 С

Резюме. Кроличьи эмбрионы в стадии восьми клеток и морул замораживали при температуре —196 °С и хранили 2—200 суток. После оттаивания определяли жизнеспособность эмбрионов в условиях in vitro и in vivo. В течение культивации in vitro стадии бластоцист достигали до 62,5% замороженных 8-клеточных эмбрионов и 81,4% замороженных морул. Из контрольной группы без замораживания продолжали свое развитие 93,2% 8-клеточных эмбрионов и 92,4% морул. Культивация in vitro позволила более надежно исключать эмбрионы, поврежденные в течение замораживания и оттаивания. В половой тракт реципиенток эмбрионы переносили или немедленно после оттаивания, или после 24-часовой культивации in vitro. Синхронный перенос замороженных кроличьих эмбрионов не давал успеха. После несинхронного переноса морул и бластоцист в яйцевод имплантировались соответственно 31,8% и 42,9% эмбрионов. После переноса бластоцист в углы матки реципиенток имплантировались 47,6% эмбрионов.

TOMUS XXVII FASCICULUS 4 1981
FOBLAN 27 (4) 225 – 288 (1981)

Folia biologica

JOURNAL OF CELLULAR AND MOLECULAR BIOLOGY

ACADEMIA PRAHA

Eur. J. Obstet. Gynecol. Reprod. Biol., 21 (1986) 187–195
Elsevier

EJO 00280

In vitro fertilization and embryo transfer of pre-ovulatory rabbit oocytes

S. Al-Hasani, * S. Trotnow, C. Sadtler and J. Hahn

Klinik für Frauenheilkunde mit Poliklinik und Hebammenschule der Universität Erlangen-Nürnberg, (Director: Prof. K.G. Ober, MD), Universitätsstrasse 21/23, D-8520 Erlangen, F.R.G.

Accepted for publication 9 August 1985

Summary

The objective of this study was to develop an effective and successful technique of in vitro fertilization in rabbits that could lead us to start treatment of infertile couples by using extracorporal fertilization.

Pre-ovulatory oocytes were harvested from follicles of virgin does 9 h after induction of ovulation by hCG injection, and incubated with in vivo capacitated sperm for 5 h. An 82% in vitro fertilization rate was achieved. The fertilized ova were cultivated for an additional 20, 44 and 68 h, and until they reached the hatched blastocyst stage. Most of these embryos (439) were transferred to 42 pseudopregnant recipients, asynchronous in the sense that they had been given hCG injections 0, 6, 12, 18, 24, 36 and 42 h later than the rabbits from which the ova had been obtained. With this type of synchronicity implantation rates of 31, 78, 52, 33, 52, 33, 51 and 10% were achieved, respectively.

One hundred and twenty-eight embryos were not transferred to recipients but cultured in Ham's F-10 medium supplemented with 20% FCS. 77% of these embryos reached the stage of hatched blastocyst. A total of 143 young were born after 28–32 days of gestation and all are fertile till the third generation.

Our results were obtained in a consecutive series of experiments, and demonstrated that in vitro fertilization can be made to work quite well in the rabbit, which in the early stages of embryogenesis has sufficient similarity to the human to make it a useful model for those wishing to acquire techniques applicable to human fertilization in vitro and embryo transfer.

in vitro fertilization; embryo transfer; synchronization; rabbit; oocytes

* *Present address*: Universitäts-Frauenklinik Bonn, Sigmund-Freud-Str. 25, D-5300 Bonn 1, F.R.G.

Introduction

Nineteen eighty-one was the year of the final breakthrough in the field of in vitro fertilization as a method of treating infertile women. About 600 children conceived in this way were born throughout the world [1–3]. Despite these successes, however, we cannot yet be satisfied with the maximum pregnancy rate presently achievable after in vitro fertilization and embryo transfer. One of the problems that still have to be solved is the 'desynchronization' between the embryo and endocrinal status in the woman – a particularly important point in human subjects where donor and recipient are identical. While, after follicle puncture, the luteal phase develops at a normal rate [4], the in vitro conditions obtaining during the cultivation of the embryo give rise to a delay in the growth of the extracorporal embryos.

The objective of this study was to investigate the extent of the 'desynchronization' for varying cultivation times, in animal experiments. For various reasons, which we have already discussed elsewhere [5], we selected the rabbit as our experimental animal. We have been working on the in vitro fertilization technique intensively for a number of years now, and have developed a reproducibly functioning in vitro fertilization model for pre-ovulatory rabbit oocytes [6]. A number of investigators [7–9] have reported on the influences of synchronicity, i.e. between the age of the transferred embryos and the endocrinal status (time elapsed after ovulation) of the recipient, on the results of embryo transfer in various laboratory animals.

Our experiments were designed to enable us to transfer pre-ovulatory rabbit oocytes, fertilized in vitro and cultured for periods varying between 24 and 72 h, to synchronous and asynchronous foster animals. In the latter animals, ovulation was induced after a varying delay vis-a-vis the oocyte donors.

Material and methods

Animals

Cross-bred does were used for the collection of follicular oocytes, the recovery of capacitated uterine sperm, and as recipients. All animals were at least 6 months old and virgins. They were caged individually for no less than 3 weeks before being used, to avoid pseudopregnancy. Males and females were housed in separate air-conditioned (21 °C) rooms. Lights were on in the rooms for 12 h during each 24-h period. Animals were fed rabbit chow and provided with water ad libitum.

Media and culture conditions

Brackett's defined medium [10] supplemented with 3 mg/ml bovine serum albumin (BSA) was used for in vitro fertilization and recovery of capacitated sperm. Ham's F-10 supplemented with 20% inactivated (30 min at 56°C) fetal calf serum (FCS) served as culture medium. Media were sterilized by positive-pressure filtration (0.22 μm millipore filters) and equilibrated with a gas phase of 5% CO_2, 5% O_2 and 90% N_2. The same gas mixture was used during incubation in an anaerobic jar inside an incubator at 37°C. To achieve a high relative humidity, the gas was bubbled through distilled water at the bottom of the anaerobic jar.

Oocyte donors

The donor females were superovulated by subcutaneous injections of 0.4 mg FSH (Burns Biotec Laboratories incorporated Omaha, Nebraska) in 4 single doses over 3 days. Ovulation was induced by an intravenous injection of 100 I.U. of hCG (Primogonyl, Schering AG, Berlin-Bergkamen) given together with the last priming dose of FSH. Ovum donors were killed 9 h later. The ovaries were excised, submerged in Brackett's medium and kept in petri dishes at 37°C. After puncturing all follicles seen over the surface of the ovaries, the oocytes were aspirated with a finely pointed glass pipette. Follicular ova in surrounding cumulus cells were washed twice in Brackett's medium and kept under described conditions in the incubator until the recovery of capacitated sperm.

Collection of capacitated spermatozoa

The ejaculates of 6–8 bucks were collected by an artificial vagina using a teaser doe. To produce capacitated spermatozoa, does were inseminated intravaginally with 4 ml of pooled semen. Just after the insemination the animals received an injection of 100 I.U. of hCG intravenously to induce ovulation. Capacitator does were laparotomized 14 h after insemination [11,12]. Both uteri were flushed with 2–3 ml of warm Brackett's medium after puncturing the uterus near the utero-tubal junction with a blunt needle. To prevent leakage of the flushing medium the cervix was clamped with an artery forceps. The fluid containing capacitated sperm was collected in petri dishes, and covered with autoclaved paraffin oil. The petri dishes were wrapped in aluminium foil to prevent direct exposure to light [13]. Then the oviducts were flushed to collect the fertilized eggs, which were cultivated in Ham's F-10 and used as controls.

Handling of the gametes

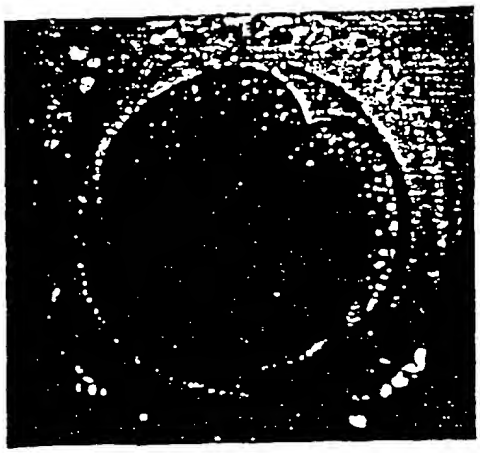
Ten to thirty follicular oocytes were added to one petri dish containing 2–3 ml capacitated sperm flushed from one side. Details of our technique have been described in a recent publication [5]. The incubation of the gametes was carried out under described conditions. Five hours later the ova were washed twice in Ham's F-10 culture medium and cultivated for a further 20–72 h.

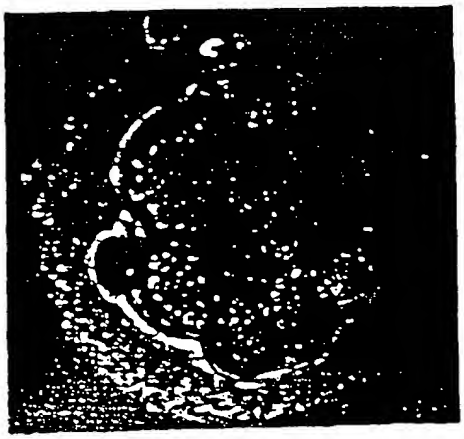
Criterion of successful in vitro fertilization

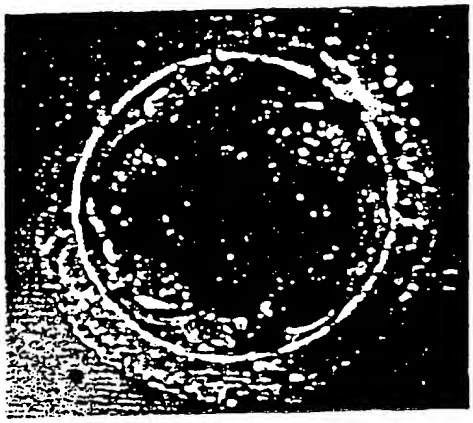
Normal cleavage to the 2- or 4-cell stage was used as the sole criterion of successful fertilization. A second polar body, sperm penetration of the zona pellucida and unequal blastomeres were not considered as criteria of successful fertilization. Adhering granulosa cells only occasionally needed to be removed mechanically to permit the blastomeres to be seen clearly on microscopy.

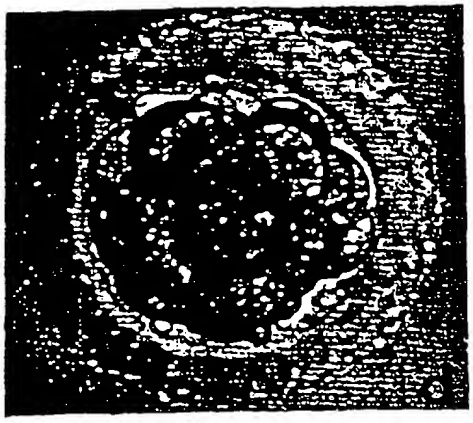
Embryo transfer

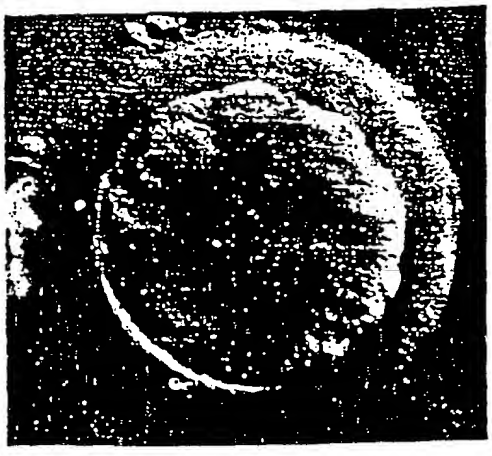
The recipients were made pseudopregnant by an intravenous injection of 100 I.U. hCG given 0, 6, 12, 18, 24, 30, 36, and 42 h after the oocyte donors received their ovulatory injection of hCG. These intervals were intended to compensate for the delayed development of the embryos due to in vitro conditions. Preimplantation embryos from the 4-cell stage to early blastocytes (Fig. 1a–f), which had been

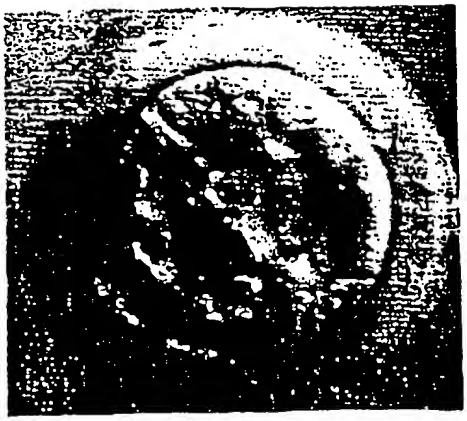












cultured for 24–72 h following in vitro fertilization, were transferred with the help of a blunt capillary glass pipette into the oviducts of the foster mothers through flank incisions.

Control of successful embryo transfer

Recipients were laparotomized after 15 days gestation in order to count the number of implantations and to distinguish between normal and retarded, apparently resorbing, fetuses. All pregnant does were allowed to deliver their offspring.

Results

Collected eggs

The number of follicles were counted by inspection of the excised ovaries. A total of 1353 (95%) ova were harvested from 1423 follicles in 82 ovaries obtained from 41 does. Among these, 166 oocytes from atretic follicles were discarded. Red-colored, unruptured follicles (folliculum haemorrhagicum) were neither counted nor aspirated. During our experiments not a single ovulation had occurred 9 h after hCG injection.

In vitro fertilization rate

An 82% in vitro fertilization rate of preovulatory oocytes recovered 9 h after hCG injection (Table I) was achieved. Owing to a lack of recipients, 167 fertilized ova could not be used for transfer experiments. These were cultured for an additional 4–5 days and 128 (77%) blastocysts developed.

Results of embryo transfer

Table II(a) contains data on the transfer of embryos, cultured for 24–26 h, to the oviducts of synchronous pseudopregnant recipients that had received their hCG injection at the same time as the donor animals. Sixteen (31%) implantations were achieved among 52 transferred embryos; only 2 resorptions occurred. Fourteen viable young were born on the 31st day of pregnancy.

The transfer results for embryos cultured for 24–26 h to recipients that had received their hCG injections 6 h later than the ovum donor are summarized in Table II(b). On the 15th day of pregnancy 54 (78%) implantations had taken place (after 69 embryos had been transferred). Among these 54 implantations 7 resorptions were seen. A total of 47 viable young were born at term.

Table II(c) contains data on the transfer of embryos cultured for 24–26 h to foster mothers that had received their hCG injection 12 h later than the donor animals. On the 15th day of pregnancy 30 (45%) fetuses had developed from 67 transferred embryos, and an additional 5 resorptions were counted. Twenty-seven viable young were born at term.

Fig. 1. In vitro fertilized embryos. (a) 4-cell-stage embryo 24 h after in vitro fertilization (i.v.f.). (b) 8-cell-stage embryo 26 h after i.v.f. (c) 16-cell-stage embryo 48 h after i.v.f. (d) A morula-stage embryo 48 h after i.v.f. (e) Late morula-stage embryo 72 h after i.v.f. (f) Early blastocyst 72 h after i.v.f.

TABLE I

In vitro fertilization results of preovulatory rabbit oocytes

Expt. No.	No. of animals	No. of collected oocytes	Fertilized oocytes 2–4- or 8-cell stage		No. of embryos cultured 4–5 days	Blastocysts	
			No.	%		No.	%
28	41	1187 *	972	82	167	128	77

* Total number of oocytes 1353; 166 oocytes of atretic follicles were discarded.

The results obtained with embryos cultured for 48 h and transferred to the oviducts of recipients that had received their hCG injection 18 h later than the oocyte donor are summarized in Table III(a). During a monitoring laparotomy on the 15th day of pregnancy, 16 (26%) fetuses had developed from 61 transferred embryos, and 4 resorptions were counted. Ten viable young were born at term.

Table III(b) shows data on the transfer of embryos cultured for 48 h, to the oviducts of pseudopregnant foster mothers that had received their hCG injection 34 h earlier. Thirty-two (52%) implantations were achieved among 61 transferred embryos; 13 resorptions occurred. Nineteen viable young were delivered on the 31st to 32nd day of gestation.

Table IV(a) contains data on the transfer of embryos cultured for 72 h to the oviducts of pseudopregnant recipients that had received their hCG injections 52 h earlier. Fifteen (33%) implantations were achieved among 46 transferred embryos. A total of 10 viable young were born on the 31st to 32nd day of gestation, and 4 resorptions occurred.

Table IV(b) shows the results of the transfer of embryos cultured for 72 h to the oviducts of pseudopregnant foster mothers that had received hCG injection 46 h earlier. Twenty-two (51%) implantations were achieved among 43 transferred embryos, and 5 resorptions occurred. At the end of the gestation period, 14 viable young were born.

TABLE II

Transfer results of in vitro fertilized rabbit oocytes cultured for 24–26 h

Recipients were given hCG (a) at the same time as, (b) 6 h and (c) 12 h later than the rabbits from whom the ova were obtained.

	No. of animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	6	52	16	31	14	27	2	4	14
(b)	6	69	54	78	47	68	7	10	47
(c)	6	67	35	52	30	45	5	7	27

Table IV(c) summarizes the results obtained with embryos cultured for 72 h and transferred to the oviducts of pseudopregnant recipients that had received their hCG injection 40 h before the transfer. On the 15th day of gestation 2 (5%) fetuses had developed among 40 transferred embryos, and 2 resorptions had occurred. Only 2 viable young were born at the end of the gestation period.

Discussion

In earlier reports, we showed that the in vitro fertilization of pre-ovulatory rabbit oocytes could be effected at our laboratory in a reproducible and reliable manner [5,6]. The results presented here with considerably greater numbers of animals confirm those obtained earlier. In summary, our relative and absolute recovery rate for oocytes, and the in vitro fertilization rates, exceed those obtained earlier, irrespective of whether the various investigators made use of preovulatory or already ovulated tubal oocytes [9,10,12,14–17]. However, this was merely an interesting 'by-product' of our investigation.

The actual purpose of this study was to determine the loss of synchronicity of rabbit oocytes fertilized in vitro, for varying times of cultivation. The answer to this

TABLE III

Transfer results of in vitro fertilized rabbit oocytes cultured for 48 h

Recipients were given hCG 18 h (a) and 24 h (b) later than the rabbit from whom ova were obtained.

	No. of Animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	6	61	20	33	16	26	4	7	10
(b)	6	61	32	52	19	31	13	21	19

TABLE IV

Transfer results of in vitro fertilized rabbit oocytes cultured for 72 h

Recipients were given hCG 30 h (a), 36 h (b) and 42 h (c) later than the rabbits from whom ova were obtained.

	No. of animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	4	46	15	33	11	24	4	9	10
(b)	4	43	22	51	17	40	5	12	14
(c)	4	40	4	10	2	5	2	5	2

question is of importance, since the loss of synchronicity following in vitro fertilization and embryo transfer is probably of considerable significance in the human situation. The animal model 'rabbit' is particularly suitable for synchronicity studies, since the time of ovulation of this reflex ovulator can be determined exactly by the injection of hormones.

Our transfer results seem to prove that the in vitro conditions apparently lead to a delay in the development of the embryos. This loss of synchronicity can be, in part, compensated by using 'asynchronous' recipient animals, that is, the foster animals are induced to ovulate somewhat later than the oocyte donors. The longer the in vitro fertilized embryos are cultivated, the greater is the loss of synchronicity. In our system of in vitro fertilization with subsequent embryo transfer, the following asynchronicities of the recipient animals proved optimal:

Cultivation duration after IVF	Optimal asynchronicity of the recipients
24 h	minus 6 h
48 h	minus 24 h
72 h	minus 36 h

Even when full advantage is taken of the optimal asynchronicities of the recipients, the transfer successes, determined by the number of implantations and viable progeny, decrease with increasing culture time.

The last two facts show that our cultivation conditions are not yet optimal. Here, we believe, is a large area for future research. We expect similar laws to apply in in vitro fertilization in human subjects, too.

Steptoe and Edwards [18] achieved their first successes only after reducing their cultivation time. The overall very modest success obtained with embryo transfer in humans is attributed, among other things, to the loss of synchronicity, which still pertains, despite a reduction in culture time.

Using our in vitro fertilization model in the rabbit, we were able to produce 143 living progeny. This is the largest number so far reported in a consecutive series in this species.

A particularly noteworthy observation is, we believe, the fact that, despite very thorough examinations and subsequent observation, we were unable to find any malformations in the progeny. These animals produced by in vitro fertilization have already reproduced in the third generation. We are using them in our 'colony' for further in vitro fertilization experiments. There is no evidence to suggest that their fertility is in any way impaired.

References

1 Edwards RG, Test-tube babies, 1981. Nature 1981; 293: 253–256.

2 Trotnow S, Kniewald T, Al-Hasani S, Becker H. Follikelpunktion, In vitro-Fertilisierung, Embryo-Transfer und eingetretene Schwangerschaft in Dyneric/HCG stimulierten Zyklen. Geburtsh Frauenheilk 1981; 41: 835–836.

3 Wood C, Trounson A, Leeton J, McKenzie JT, Buttery B, Webb J, Wood J, Jessup D. A clinical assessment of nine pregnancies obtained by in vitro fertilization and embryo transfer. Fertil Steril 1981; 35: 502–508.
4 Trotnow S, Becker H, Kniewald T, Al-Hasani S, Mulz D. Luteal phase function following oocytes aspiration, in vitro fertilization, and embryo transfer in the Clomid/hCG stimulated cycle. Arch Gynec 1982; 231: 171–176.
5 Trotnow S, Al-Hasani S, Sadtler C. Experience with in vitro fertilization of follicular rabbit oocytes and embryo transfer. Arch Gynecol 1981; 231: 41–50.
6 Al-Hasani S. In vitro-Befruchtungsversuche mit praeovulatorischen Kaninchenei-zellen. Naturwissensch. Diss. Tierärztl. Hochschule, Hannover, 1980.
7 Binkerd PE, Anderson GB. Transfer of cultured rabbit embryos. Gamete Res 1971; 2: 65–73.
8 Schneider U, Jakovac M, Hahn J. Die Bedeutung der Synchronisation zwischen Eispender und -empfänger dargestellt am Kaninchenmodell. Fortschr Fertilitätsforsch 1977; V: 168–170.
9 Seidel GE, Bowen RA, Kane MT. In vitro fertilization, culture, and transfer of the rabbit ova. Fertil Steril 1976; 27: 861–870.
10 Brackett BG, Williams WL. Fertilization of rabbit ova in a defined medium. Fertil Steril 1968; 19: 144–155.
11 Hahn J, Sulzer H, Schneider U. Ergebnisse von in vitro-Befruchtungsversuchen mit Kaninchenei-zellen. A – Einflüsse der Samenzellkonzentration und der Kapazitationsdauer. Zuchthyg 1974; 9: 178–184.
12 Hahn J, Sulzer H, Schneider U. Ergebnisse von in vitro-Befruchtungsversuchen mit Kaninchenei-zellen. B – Der Einfluß des Eizellalters nach der Ovulation (P.O.) auf die Entwicklung der Eizellen. Zuchthyg 1975; 10: 24–28.
13 Fraser LR, Dandekar PV. The effect of aging on in vitro fertilization of rabbit eggs and subsequent embryonic development. J Exp Zool 1973; 184: 303–312.
14 Brackett BG, Killen DE, Peace MD. Cleavage of rabbit ova inseminated in vitro after removal of follicular cells and zona pellucidae. Fertil Steril 1971; 22: 816–826.
15 Brackett BG, Mills JA, Jeitles GG. In vitro fertilization of rabbit ova recovered from ovarian follicles. Fertil Steril 1972; 23: 898–909.
16 Kubo H. In vitro fertilization of rabbit ova in chemically defined medium. In: Ebling FJG, Henderson IW, eds. Biological and clinical aspects of reproduction. Amsterdam, Excerpta Media, 1976; 161–164.
17 Mills IA, Jeitler GG, Brackett BG., Embryo transfer following in vitro and in vivo fertilization of rabbit ova. Fertil Steril 1973; 24: 602–608.
18 Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978; ii: 366.

european journal of obstetrics & gynecology and reproductive biology

EOGRAL 21 (3) 125–198

Vol. 21, No. 3, March 1986

Cited in: Current Contents/Clinical Practice, Index Medicus, Excerpta Medica, Pascal M

ELSEVIER

THIS PAGE BLANK (USPTO)

A cloned horse born to its dam twin

A birth announcement calls for a rethink on the immunological demands of pregnancy.

Several animal species, including sheep, mice, cattle, goats, rabbits, cats, pigs and, more recently, mules[1] have been reproduced by somatic cell cloning, with the offspring being a genetic copy of the animal donor of the nuclear material used for transfer into an enucleated oocyte. Here we use this technology to clone an adult horse and show that it is possible to establish a viable, full-term pregnancy in which the surrogate mother is also the nuclear donor. The cloned offspring is therefore genetically identical to the mare who carried it, challenging the idea that maternal immunological recognition of fetal antigens influences the well-being of the fetus and the outcome of the pregnancy.

We derived equine fibroblast cell lines from skin biopsies taken from one Arabian thoroughbred male and one Haflinger female. Oocytes were obtained by *in vitro* maturation of follicular oocytes recovered from equine ovaries retrieved from abbatoirs. After removal of the nucleus and zona material, embryos were reconstructed by cell fusion with the fibroblasts. Only fused embryos (97%) were activated and cultured *in vitro* to the blastocyst stage; these were then transferred into recipient mares (see supplementary information).

We cultured 513 and 328 successfully fused reconstructed embryos from the male and the female cell lines, respectively. In total, 22 blastocysts developed, 8 from the male and 14 from the female cell line. Of these, eight blastocysts from the male cell line and nine from the female line were transferred non-surgically into nine recipients. Two of the cloned embryos that were reconstructed with female cells were transferred into the Haflinger mare from which the cell line was derived.

Overall, four single pregnancies were diagnosed by ultrasonography after 21 days of gestation. Two were lost soon afterwards and one aborted at 187 days of gestation; the fourth, however, was carried to term (336 days) and a healthy female foal was born naturally on 28 May 2003 (birth weight, 36 kg). Remarkably, this cloned foal was born from the same Haflinger mare who was the original cell-line donor (Fig. 1).

To confirm the genetic identity of the foal and her mother, we analysed DNA from blood leukocytes from both animals and from the placenta. Comparison of 12 equine-specific microsatellite loci (StockMarks kit, Perkin-Elmer, Applied Biosystems) confirmed that DNA from the foal and from the placenta was identical to that of the recipient mare.

Our cloning procedure was relatively efficient, as one live foal was produced from four pregnancies, although there was high developmental failure from the cleavage stage to blastocyst (8 of 467 and 14 of 286 developed in the male and female cell lines, respectively) and early implantation. This success was aided by advances in assisted reproduction in the horse[2], particularly at the oocyte-activation stage, when protein synthesis and phosphorylation must both be inhibited[3], and in the refinement of the zona-free manipulation technique[4,5].

The remarkable birth of a live foal from its genetically identical recipient is at odds with the idea that the maintenance of gestation depends on immunological recognition of the pregnancy by the mother, based on evidence that abortion can result from inadequate recognition of fetal antigens[6].

Furthermore, our result adds the horse to the list of mammals that have so far been successfully cloned from an adult somatic cell. In principle, cloning could enable gelding champions to contribute their genotype to future generations, as well as opening up an opportunity to verify the reproducibility of traits such as character and sporting performance.

Cesare Galli, Irina Lagutina, Gabriella Crotti, Silvia Colleoni, Paola Turini, Nunzia Ponderato, Roberto Duchi, Giovanna Lazzari

Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, 26100 Cremona, Italy

Figure 1 The cloned foal, named Prometea, with its genetically identical mother seven days after birth. The cells used for cloning were derived from a skin biopsy of the mare. The enucleated oocytes were obtained by *in vitro* maturation and removal of the nucleus from oocytes recovered from horse ovaries from an abattoir. After embryo reconstruction by micromanipulation and cell fusion, the cleaved embryos were cultured *in vitro* to the blastocyst stage. Two of the derived embryos were transferred into the mare donor of the cell line. A single pregnancy was established and was carried to term with the birth of a female foal who is genetically identical to her surrogate mother.

e-mail: cesare@galli2.191.it

1. Woods, G. L. *et al. Science* doi:10.1126/science.1086743 (2003).
2. Galli, C. *et al. Theriogenology* 58, 705–708 (2002).
3. Lazzari, G., Mari, G. & Galli, C. *J. Reprod. Fertil. Abstr. Ser.* 28, 73 (2002).
4. Lagutina, I. *et al. Theriogenology* 59, 269 (2003).
5. Wilmut, I. *Cloning and Stem Cells* 5, 1–2 (2003).
6. Szekeres-Bartho, J. *Int. Rev. Immunol.* 21, 471–495 (2002).

Supplementary information accompanies this communication on *Nature*'s website.

Competing financial interests: declared none.

Lithium-ion batteries

Runaway risk of forming toxic compounds

Lithium-ion batteries are stabilized by an ultrathin protective film that is 10–50 nanometres thick and coats both electrodes. Here we artificially simulate the 'thermal-runaway' conditions that would arise should this coating be destroyed, which could happen in a battery large enough to overheat beyond 80 °C. We find that under these conditions the reaction of the battery electrolyte with the material of the unprotected positive electrode results in the formation of toxic fluoro-organic compounds. Although not a concern for the small units used in today's portable devices, this unexpected chemical hazard should be taken into account as larger and larger lithium-ion batteries are developed, for example for incorporation into electric-powered vehicles.

Lithium-ion cells are now ubiquitous in consumer electronics. The negative electrode consists of lithium in graphite and the positive electrode contains lithium and a transition metal such as cobalt, nickel or manganese. However, with a lead voltage of about 4 volts, no electrolyte is thermodynamically stable towards either electrode, so the operation of these batteries relies on a combination of the ethylene carbonate and $LiPF_6$ ($LiBF_4$)-based electrolyte producing a continuous film that ensures sufficient ionic conductivity and provides electronic insulation to protect the electrodes.

Above 80 °C, thermal runaway can occur spontaneously as a result of the break-up of this protective film. Today's commercial batteries are checked by electronic monitoring of individual cells and by a self-clogging polyolefin separator to prevent overheating,

daily for thirty two days during winter months of January and February. Estrus was detected by visual symptoms, parading a vasectomized bull twice daily and confirmed by monitoring plasma progesterone concentrations. Progesterone concentrations were determined by a simple, direct RIA. The sensitivity of assay was 8 pg/tube. The intra- and inter-assay coefficients of variation were 13.4 and 16.9 percent, respectively. Plasma progesterone concentrations increased from 0.40 ± 0.02 and 0.47 ± 0.03 ng/ml during periestrus phase to 0.74 ± 0.10 and 0.94 ± 0.08 ng/ml during early luteal phase and then further ($P<0.05$) to 1.94 ± 0.22 and 1.39 ± 0.13 ng/ml during midluteal phase following which declined ($P<0.05$) to 0.63 ± 0.16 and 0.95 ± 0.19 ng/ml during late luteal phase in cows exhibited overt estrus and silent estrus, respectively. Plasma progesterone concentrations increased from 0.42 ± 0.02 and 0.38 ± 0.02 ng/ml during periestrus phase to 0.66 ± 0.12 and 0.51 ± 0.07 ng/ml during early luteal phase and then further ($P<0.01$) to 1.55 ± 0.33 and 1.30 ± 0.13 ng/ml during midluteal phase following which declined ($P<0.01$) to 1.18 ± 0.27 and 0.66 ± 0.13 ng/ml during late luteal phase in buffaloes exhibited overt estrus and silent estrus, respectively. Overall plasma progesterone levels were lower in cows and buffaloes that exhibited silent estrus compared to overt estrus and might be responsible for poor expression of estrus.

73. Synergic effect of cycloexemide and 6-DMAP on activation of equine and bovine oocytes.

G Lazzari[1], G Mari[2] & C Galli[1], [1]Laboratorio di Tecnologie della Riproduzione, Via Porcellasco 7/F, 26100 Cremona, Italy; [2]Facoltà di Medicina Veterinaria, Università degli Studi di Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Italy.

Cycloheximide (CHX) and 6-DMAP at concentration of 10μg/ml and 2mM respectively are used routinely for the activation of bovine oocytes in association with ionomycin. By contrast, little information is available regarding their effect on the equine oocyte and also their use in combination. For this study oocytes were matured *in vitro* for 24h (bovine) or 28h (equine) in medium TCM 199 supplemented with 10% FCS (bovine) or 10% Serum Replacement (equine, Life Technologies) and 0.1 IU of LH and FSH. Matured oocytes were activated with 5 μM ionomycin for 4 minutes, rinsed and transferred in medium SOF-AA-BSA supplemented with CHX or DMAP or for 4h. Then they were rinsed twice and transferred in medium without inhibitors and 10 to 14 h later they were fixed and stained with Hoechst. All experiments were done in three replicates. For the equine oocytes the activation rate was 30.6% (11/36) for CHX (10μg/ml), 60% (15/25) for DMAP (2mM) and 93.1% (27/29) for CHX + DMAP indicating a clear synergic effect between the compounds (chi square test, $p<0.05$). For the bovine oocytes we tested also lower concentrations. When the compounds were used alone we observed a progressive reduction of the activation rate: for CHX 69% (30/44, 10 μg/ml), 47% (21/45, 5 μg/ml), 46% (19/41, 2.5 μg/ml) and for DMAP 100% (30/30, 2mM), 93% (26/28, 1mM), 43% (13/30, 0.5mM). However when the inhibitors were used together the activation rate was 100% at the two combinations tested: 100% (31/31, 5μg/ml CHX + 1mM DMAP) and 100% (35/35, 2.5μg/ml CHX + 0.5mM DMAP). Comparing these data with those obtained with the inhibitors used separately at 2.5μg/ml for CHX and 0.5mM for DMAP we found a significant increase in activation rate (46% and 43% *versus* 100%, chi square test, $p<0.05$). This study demonstrates that CXH and DMAP act in a synergic manner to induce oocyte activation both in equine and in bovine.

74. Effect of *in vitro* maturation on the expression of the peroxiredoxin family of antioxidant enzymes in bovine oocyte-cumulus complexes.

G Leyens[1], B Knoops[2] & I Donnay[1]. [1]Unité des Sciences Vétérinaires ; [2]Laboratoire de Biologie Cellulaire, Institut des Sciences de la Vie, Universtité catholique de Louvain, Place Croix du Sud 3, 1348 Louvain-la-Neuve, Belgium.

The expression of peroxiredoxins (PRDX1 to 6), a recently characterized family of antioxidant enzymes, has been studied in bovine oocyte-cumulus complexes (COCs) before and after *in vitro* maturation by multiplex RT-PCR.

Pools of COCs, denuded oocytes and cumulus cells have been frozen at –80°C. Total RNA was extracted in presence of glycogen (2%). The reverse transcription was done using Superscript II (Life Technologies), while Ex Taq (TaKaRa) was used for the multiplex and standard polymerase chain reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

The six peroxiredoxins were detected by multiplex RT-PCR both in COCs and denuded oocytes before and after maturation. In cumulus cells, all peroxiredoxins were also expressed before and after maturation, except PRDX6 that was not detectable by multiplex RT-PCR before maturation. Standard PCR confirmed an important overexpression of PRDX6 in cumulus cells after maturation.

Our results show that most peroxiredoxins are expressed in bovine cumulus cells and oocytes. Furthermore, transcription of PRDX6 is induced in cumulus cells during maturation. PRDX6 is the only peroxiredoxin with phospholipase A2 activity, which contributes with cyclo-oxygenase to the synthesis of prostaglandins. Since prostanoids are a major factor involved in cumulus expansion, PRDX6 could play an important role in COC maturation.

G Leyens is a Research Fellow of the Fonds National de la Recherche Scientifique de Belgique.

75. Effect of ovarian status on maturation potential of sheep oocytes.

RM Garcia-Garcia, A Gonzalez-Bulnes, V Dominguez & MJ Cocero. Departamento de Reproduccion Animal, INIA. Avda. Puerta de Hierro s/n. 28040-Madrid, Spain.

Number of viable embryos in superovulated ewes are affected by presence of large follicles (≥6mm in size) and/or Corpus luteum at start gonadotrophin treatments (Gonzalez-Bulnes *et al.* 1999, JRF Abstracts 24: 6; Gonzalez-Bulnes *et al.* 2000, 2000 ESDAR Newsletter: 14), suggesting that a proportion of the smaller follicles stimulated to grow and ovulate by exogenous FSH may be in early stages of atresia and subsequently developmental competence of its oocytes should be compromised. This study have tested this hypothesis by assessing possible effects from ovarian status at start FSH treatment on the ability of oocytes from smaller follicles to undergo nuclear maturation *in vitro*. A

1: Dev Biol. 1994 Dec;166(2):729-39.

ELSEVIER FULL-TEXT ARTICLE

Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion.

Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL.

Department of Meat and Animal Science, University of Wisconsin, Madison 53706.

We have examined the response of bovine oocytes matured in vitro for 24 hr to parthenogenic activation using compounds that increase intracellular calcium (ionomycin) or inhibit protein phosphorylation (6-dimethylaminopurine, DMAP). Treatment with ionomycin alone caused resumption of meiosis (57.8 +/- 7.8%) but not pronuclear formation (8.9 +/- 7.3%). DMAP alone did not cause resumption of meiosis or pronuclear formation. Sequential treatment with ionomycin (5 microM for 4 min) immediately followed by DMAP (1.9 mM for 5 hr) resulted in activation that led to pronuclear formation (80.5 +/- 13.1%). Completion of meiosis, however, was bypassed as evidenced by only one polar body and one pronucleus present in activated parthenogenones. It was necessary to incubate the oocytes for at least 3 hr in DMAP to obtain high rates of activation (76.6 +/- 9.8%) and development to blastocysts (21.1 +/- 1.5%). Temporal separation of the two treatments resulted in a decrease in oocytes with one pronucleus and one polar body (uniformly diploid parthenogenones) and an increase in a mixture of diploid and haploid parthenogenones since DMAP was capable of causing transition to interphase of all chromatin configurations after anaphase commenced and prior to metaphase arrest. Parthenotes produced with ionomycin and DMAP that developed to the blastocyst stage had high cell numbers (70 to 88 cells) and were able to cause extended cycles in 33.3% of recipient cattle after nonsurgical transfer to the uterus. Response of the bovine oocyte arrested in metaphase II to different activation stimuli was also found to show age-dependent changes in pattern of activation response and developmental competence.

NUCLEAR TRANSFER IN HORSES

I. Lagutina, G. Crotti, S. Colleoni, N. Ponderato, R. Duchi, G. Lazzari, and C. Galli

Laboratorio di Tecnologie della Riproduzione, Cremona, Italy

In this study we investigated the developmental ability of horse embryos after nuclear transfer (NT) with cumulus cells or adult skin fibroblasts. Equine oocytes with compact and expanded cumulus were matured for 20–22 h in TCM199 with 10% FCS, 0.1 IU/ml LH and 0.1 IU/ml FSH, decumulated and enucleated. Cumulus cells for NT were obtained by trypsinisation from maturing oocytes with compact cumulus. Fibroblasts of passage 1–6 were cultured in TCM199/DMEM with 10% FCS to establish a confluent monolayer or were serum starved for 1–4 days. NT-embryos were obtained: (1) by transfer of a cell under the zona pellucida and fusion in 0.3 M mannitol by double pulse of 30 μs DC 2.4 kV/cm; or (2) by attachment of zona pellucida free (0.5% pronase) oocytes to a single cell in TCM199 with 200 μg/ml of lectin (PHAP) and fusion by double pulse of 30 μs DC 1.2 kV/cm. The fusion rate of zona-intact ooplasts with cumulus cells was 69%, while 100% of zona-free ooplasts fused with cumulus cells and 97% with fibroblasts ($P \leq 0.05$). One 2 h post fusion embryos were activated by 5 μM ionomycin for 4 min and incubated in the mixture of 5 μg/ml cycloheximide and 1 mM 6-DMAP in SOFaa for 4 h. Embryos were cultured in SOFaa in 5% CO_2, 5% O_2 at 38.5 °C. The data were compared by Chi-square test. The cleavage rate of zona-free NT-embryos (84–88%) did not depend on quality of oocyte cumulus or origin of nuclei and was significantly higher than of zona-intact NT-embryos with cumulus cell nuclei (69.2%, $P \leq 0.05$). However, this difference could be due to technical difficulties. Further work is needed to confirm it. There were 1.3–1.9% of NT-embryos with cumulus cell nuclei and 3.8–4.4% of NT-embryos with fibroblast nuclei that formed blastocysts on Day 8. This study shows that the development of NT-embryos to the blastocyst stage is not affected by the morphology of the oocyte cumulus before maturation and by the presence/absence of zona pellucida.

Nuclear donor	Cumulus morphology	Zona pellucida	Number of fused embryos	Cleaved (%)	Blastocysts (%)
Cumulus cells	Compact	+	78	54 (69.2)[a]	1 (1.3)
	Expanded	–	104	88 (84.6)[b]	2 (1.9)
Adult fibroblasts	Compact	–	205	180 (87.8)[b]	9 (4.4)
	Expanded	–	52	45 (86.5)[b]	2 (3.8)

Generation of Fertile Cloned Rats by Regulating Oocyte Activation

Qi Zhou,[1,2] Jean-Paul Renard,[1]* Gaëlle Le Friec,[3] Vincent Brochard,[1] Nathalie Beaujean,[1] Yacine Cherifi,[3] Alexandre Fraichard,[3] Jean Cozzi[3]

The rat is a reference animal model for physiological studies and for the analysis of multigenic human diseases such as hypertension, diabetes, and neurological disorders (*1*). Genetic manipulation in the rat is hampered by the lack of suitable technologies such as embryonic stem cells (ES), which are routinely used to generate targeted mutations in the mouse. Cloning through somatic cell nuclear transfer (SCNT) is a potential alternative approach in species for which ES technologies are unavailable. However, all previous efforts to clone rats have been unsuccessful, with developmental arrest at implantation stage [(*2*) and references therein].

The fine-tuned coordination between nuclear transfer and timing of oocyte activation is critical to the outcome of somatic cloning. This coordination is hampered in the rat because almost all the oocytes spontaneously, although abortively, activate within 60 min of their removal from oviducts (*3*). Such rapid but incomplete activation process is not encountered in other cloned species. To allow embryo reconstruction before the onset of oocyte activation, we initially developed a one-step SCNT procedure for the rapid substitution of the endogenous meiotic metaphase nucleus by an exogenous mitotic one. This latter nucleus was isolated from synchronized cultured fetal CD–Sprague Dawley fibroblasts [12.5 days post coitum (dpc)]. Individual mitotic nuclei were injected into a recipient OFA–Sprague Dawley oocyte, from which the meiotic metaphase nucleus was withdrawn while removing the micropipette from cytoplasm after injection. However, within 30 min after recovery, 70% of oocytes showed clear morphological evidence of spontaneous release from the second meiotic metaphase arrest (oocyte metaphase MII) (Fig. 1A). When activation of cloned embryos (Fig. 1B) was induced and maintained by exposure (2 hours) to a cdc2-specific kinase inhibitor (butyrolactone, 150 μM) (*4*), 201 of 221 reconstructed embryos expelled the polar body and subsequently divided into two-cell embryos. Their transfer into OFA–Sprague Dawley foster mothers (11 recipients, 221 embryos) resulted in nine implantation sites but no fetal development.

Forty percent of oocytes selected for SCNT had been already activated, as evidenced by disjoined sister chromatids moving to opposite poles (Fig. 1C). These observations strongly supported the view that, despite rapid manipulation, most of the oocytes were not suitable for cloning. Because activation is triggered by the inactivation of maturation promoting factor (MPF) activity through a proteasome-mediated cyclin degradation pathway, we used MG132, a protease inhibitor that reversibly blocks the first meiotic metaphase-anaphase transition in the rat (*5*). We found that this drug also reversibly stabilized most oocyte MII metaphases for up to 3 hours [77% (Fig. 1D)].

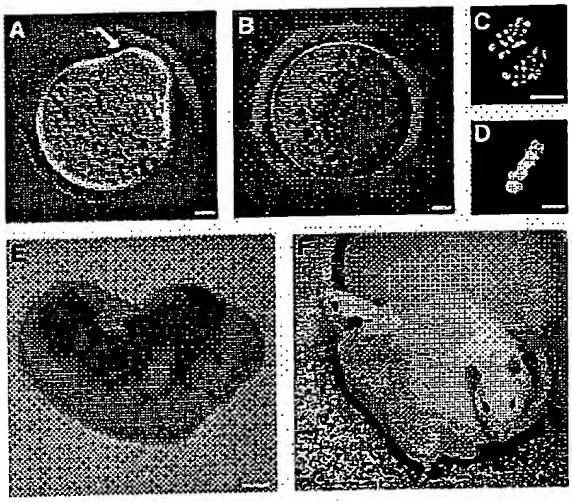


Fig. 1. (**A**) A freshly recovered rat oocyte with a marked cytoplasmic protrusion (arrow) revealing an ongoing activation process. (**B**) A rat oocyte used for micromanipulation. (**C**) DNA status of oocytes after recovery in standard conditions reveals the presence of two separate sets of chromatids, whereas after recovery in MG132 supplemented medium (**D**) a stabilized metaphase plate is revealed. (**E**) Normal cloned fetus at 14.5 dpc and (**F**) two adult cloned male rats obtained using MG132 supplemented medium. Bar: (A) to (D) 10 μm; (E) 1 mm.

We then collected oocytes in the presence of MG132 (5 μM) (*4*) as described. SCNT was performed within 30 min of drug removal. Eight hundred seventy-six embryos were implanted into 12 pseudopregnant foster female rats. At 12.5 dpc, the females were sacrificed, and four females contained 16 fetuses. Thirteen of the fetuses, obtained from three females, were viable with beating hearts (Fig. 1E). In the next series of experiments, we transplanted 129 cloned embryos into two foster mothers and allowed them to go to term. Only one foster mother contained viable fetuses, and this animal delivered three live male pups of fibroblast origin as unambiguously demonstrated by microsatellite marker analysis (*4*). One normal-sized pup (5.9 g) died a few hours after birth. The other two pups grew to sexual maturity (Fig. 1F) and generated normal progeny. We have also obtained normal progenies (in terms of size, weight, and development) from two additional cloned female rats, demonstrating the potential of the technique for the development of fertile rat lines of both sexes [supporting online material (SOM) Text].

Our data highlight the importance of adapting the SCNT procedure to oocyte physiology for successful cloning. Recently, random mutagenesis has been proposed to generate knockout rats (*6*). However, our results pave the way for more extensive genetic modifications such as conditional knock-out and gene replacement, which are required to produce relevant models of human diseases.

References and Notes

1. H. J. Jabob, A. Kwitek, *Nature Rev. Genet.* **3**, 33 (2002).
2. M. Hirabayashi *et al.*, *Cloning Stem Cells.* **5**, 35 (2003).
3. M. Zernicka-Goetz, *Mol. Reprod. Dev.* **28**, 169 (1991).
4. Materials and Methods are available as supporting material on *Science* Online.
5. L. B. Josefsberg *et al.*, *Biol. Reprod.* **62**, 1270 (2000).
6. Y. Zan *et al.*, *Nature Biotechnol.* **21**, 645 (2003).
7. Funding was provided by Institut National de la Recherche Agronomique (INRA) and genOway Company. We thank P. Hardy (DVM, Charles River Laboratories, France) and C. Szpirer (ULB) for their contribution to rat genotype analysis. We also acknowledge P. Adenot for use of the INRA confocal facility and B. Nicolas for technical help during the preparation of this manuscript.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1088313/DC1
Materials and Methods
SOM Text
Fig. S1
Tables S1 to S3
References

23 June 2003; accepted 22 September 2003
Published online 25 September 2003;
10.1126/science.1088313
Include this information when citing this paper.

[1]Developmental Biology and Reproduction Unit, INRA, Jouy en Josas, France. [2]Institute of Zoology, CAS, Beijing, People's Republic of China. [3]genOway, Lyon, France.

*To whom correspondence should be addressed. E-mail: renard@jouy.inra.fr

17. Kimura, Y. & Yanagimachi, R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121, 2397–2405 (1995).
18. Eppig, J., Wigglesworth, K., Pendola, F. & Hirao, Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol. Reprod.* 56, 976–984 (1997).
19. Chatot, C. L., Lewis, J. L., Torres, I. & Ziomek, C. A. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol. Reprod.* 42, 432–440 (1990).
20. Erickson, R. P., Zwigman, T. & Ao, A. Gene expression, X-inactivation, and methylation during spermatogenesis: the case of *Zfa*, *Zfx* and *Zfy* in mice. *Mol. Reprod. Dev.* 35, 114–120 (1993).
21. Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* 55, 789–795 (1996).
22. Kono, T., Sotomaru, Y., Sato, Y. & Nakahara, T. Development of androgenetic mouse embryos produced by *in vitro* fertilization of enucleated oocytes. *Mol. Reprod. Dev.* 34, 43–46 (1993).
23. Bos-Mikich, A., Whittingham, D. G. & Kones, K. T. Meiotic and Mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* 182, 172–179 (1997).
24. Dietrich, W. *et al.* A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics* 131, 423–447 (1992).
25. Taylor, B. A. & Rowe, L. A mouse linkage testing stock possessing multiple copies of the endogenous ecotropic murine leukemia virus genome. *Genomics* 5, 221–232 (1989).
26. Johnson, K. R., Cook, S. A. & Davisson, M. T. Chromosomal localization of the murine gene and two related sequences encoding high-mobility-group 1 and Y proteins. *Genomics* 12, 503–509 (1992).

Acknowledgements. This study was supported in part by grants from the National Institutes of Health, ProBio America Inc., and fellowships from the Japanese Society for the Promotion of Science (T.W.) and European Molecular Biology Organization (A.C.F.P.). We thank H. Tateno for chromosome analysis, Y. Nakamura for help with DNA fingerprinting, H. Kishikawa, T. Kasai and R. Kleinfeld for assistance in preparing this manuscript, and J. Eppig for help and advice.

Correspondence and requests for materials should be addressed to R.Y.

Defects in somite formation in *lunatic fringe*-deficient mice

Nian Zhang & Thomas Gridley

The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609-1500, USA

Segmentation in vertebrates first arises when the unsegmented paraxial mesoderm subdivides to form paired epithelial spheres called somites[1,2]. The Notch signalling pathway is important in regulating the formation and anterior–posterior patterning of the vertebrate somite[3–7]. One component of the Notch signalling pathway in *Drosophila* is the *fringe* gene, which encodes a secreted signalling molecule required for activation of Notch during specification of the wing margin[8–11]. Here we show that mice homozygous for a targeted mutation of the *lunatic fringe* (*Lfng*) gene, one of the mouse homologues[12,13] of *fringe*, have defects in somite formation and anterior–posterior patterning of the somites. Somites in the mutant embryos are irregular in size and shape, and their anterior–posterior patterning is disturbed. Marker analysis revealed that in the presomitic mesoderm of the mutant embryos, sharply demarcated domains of expression of several components of the Notch signalling pathway are replaced by even gradients of gene expression. These results indicate that *Lfng* encodes an essential component of the Notch signalling pathway during somitogenesis in mice.

The *Lfng* gene is expressed during somitogenesis in mice in a dynamic pattern that suggests a possible role for *Lfng* in regulating somite formation and establishing somite borders[12,13]. To analyse the role of the *Lfng* gene during embryogenesis, we constructed a targeting vector that deleted 0.7 kilobases (kb) of genomic sequence encoding the putative signal peptide and proprotein region of the Lfng protein, and replaced the deleted sequence with the *lacZ* gene of *Escherichia coli* (Fig. 1a, b). Mice heterozygous for the $Lfng^{LacZ}$ mutant allele were viable and fertile. The pattern of RNA expression from the $Lfng^{LacZ}$ mutant allele was identical to that of *Lfng* RNA expression, but expression of β-galactosidase protein from the $Lfng^{LacZ}$ mutant allele was not useful as a marker for visualizing the normal pattern of *Lfng* expression during somitogenesis owing to the perdurance of the β-galactosidase protein and the dynamic nature of the *Lfng* expression pattern (Fig. 1c–e).

At birth, $Lfng^{LacZ}$ homozygous neonates had a shortened trunk with a rudimentary tail (Fig. 2a). Some $Lfng^{LacZ}$ homozygous neonates died within a few hours of birth, apparently from respiratory difficulties due to malformed rib cages (see below), but other, less severely affected, $Lfng^{LacZ}$ homozygotes could survive to adulthood. Analysis of stained skeletal preparations revealed substantial defects in formation of the vertebral column and ribs in the $Lfng^{LacZ}$ homozygotes (Fig. 2b, c). The regular metameric pattern of the vertebrae was disrupted along the entire longitudinal axis. The ribs of the $Lfng^{LacZ}$ homozygotes were bifurcated and fused, and some ribs were detached from the vertebral column (Fig. 2c).

$Lfng^{LacZ}$ homozygous mutant embryos could be distinguished

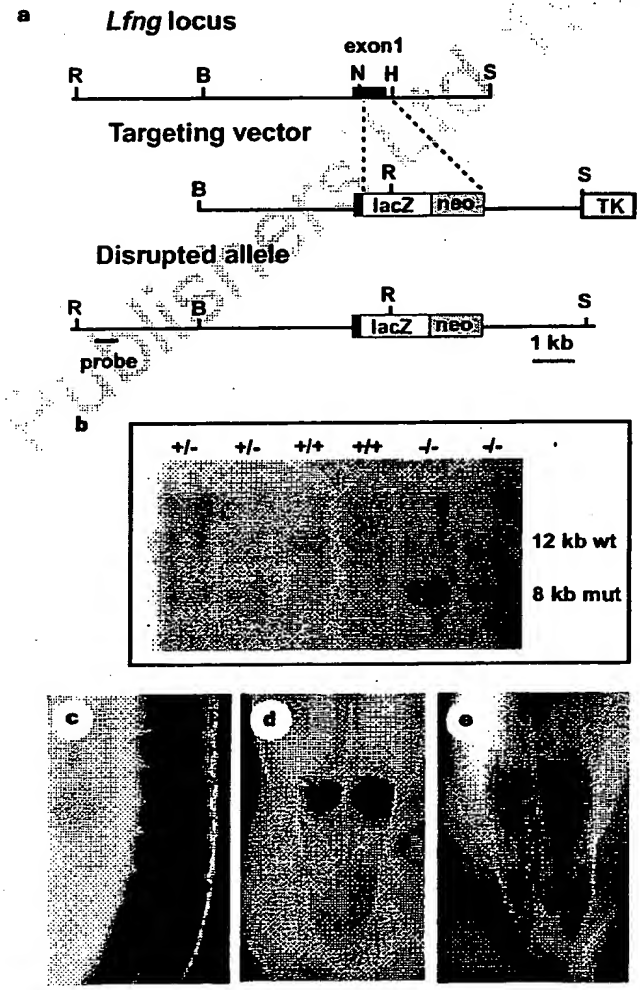


Figure 1 Targeted disruption of the *Lfng* gene. **a**, Targeting scheme. The top line shows the genomic organization of a portion of the *Lfng* gene; the middle line shows the structure of the targeting vector. A 0.7-kb deletion was created which removes most of exon 1, replacing it with the *lacZ* gene and a *neo* cassette. At the bottom is the predicted structure of the *Lfng* locus following homologous recombination of the targeting vector. The probe used for Southern blot analysis in **b** is indicated. Restriction enzymes: B, *Bam*HI; H, *Hin*dIII; N, *Not*I; R, *Eco*RV; S, *Sal*I. **b**, DNA isolated from embryos of the intercross of $Lfng^{LacZ}$/+ heterozygous mice was digested with *Eco*RV, blotted, and hybridized with the indicated probe. Genotypes of progeny are indicated at the top of each lane. **c**, β-Galactosidase expression in $Lfng^{LacZ}$/+ heterozygous embryos revealed that, unlike *Lfng* RNA, β-galactosidase protein was expressed throughout the rostral presomitic mesoderm and the most recently formed somites. **d**, *In situ* hybridization of a $Lfng^{LacZ}$/+ heterozygous embryo with a *lacZ* probe revealed the same banding pattern in the presomitic mesoderm as is observed with a *Lfng* probe, demonstrating that the constitutive expression observed in **c** is due to perdurance of β-galactosidase. **e**, *In situ* hybridization of a $Lfng^{LacZ}$ homozygous mutant embryo with a *lacZ* probe revealed that the stripe of *lacZ* RNA expression is expanded and is more diffuse than in $Lfng^{LacZ}$/+ heterozygous embryos. In **c**–**e**, posterior is at the bottom. **c**, Sagittal view; **d**, **e**, dorsal views.

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6143–6147, June 1993
Developmental Biology

Production of calves by transfer of nuclei from cultured inner cell mass cells

(bovine embryonic stem cells/nuclear transfer/totipotency)

MICHELLE SIMS AND N. L. FIRST*

Department of Meat and Animal Science, University of Wisconsin, Madison, WI 53706

Contributed by N. L. First, March 19, 1993 (revised manuscript received April 18, 1994)

ABSTRACT We report here the isolation and *in vitro* culture of bovine inner cell mass (ICM) cells and the use of ICM cells in nuclear transfer to produce totipotent blastocysts that resulted in calves born. Of 15 cell lines represented in this study, 13 were derived from immunosurgically isolated ICM of 3 *in vitro* produced day 9–10 bovine blastocysts, while 2 lines were derived from single blastocysts. Approximately 70% of attempted cell lines became established cell lines when started from 3 ICMs. The ability to establish cell lines was dependent on the number of ICMs starting the line. Sire differences were noted in the ability of ICMs to establish cell lines and to form blastocysts. The cell lines were cultured as a low cell density suspension in the medium CR1aa plus selenium, insulin, and transferrin (SIT) and 5% fetal calf serum (FCS) for 6–101 days before use in nuclear transfer, at which time some had multiplied to more than 2000 cells. If allowed to aggregate, cells of established cell lines formed embryoid bodies. A total of 659 nuclear transfer clones were made by fusing the ES cells into enucleated oocytes with polyethylene glycol; 460 of these fused, based on cleavage (70%). After culture of the clones for 7 days *in vitro* in CR1aa/SIT/5% FCS, 109 (24%) of those fused became blastocysts. Thirty-four blastocysts were transferred into uteri of 27 cows, and 13 cows (49%) became pregnant. Four of the 13 cows gave birth to 4 normal calves. DNA typing showed the calves to be derived from the respective sires of the cell lines. The calves were derived from cultures of less than 28 days.

The isolation and multiplication in culture of totipotent embryonic stem (ES) cells have value in providing a large population of identical cells for use by nuclear transfer to produce clonal offspring (1). ES cells also provide a mechanism for gene transfer by transfection, infection, or injection of genes into the cells (2–6). After insertion of a selectable marker, the transgenic cells can be separated and used either by chimerization into a blastocyst or through use as donor cells in nuclear transfer to produce transgenic offspring (5–7). In addition, homologous recombination techniques can be used with cultured ES cells to add or delete genes at specific sites in the genome (8–11).

All of the above have been accomplished only with ES cells of mice (6, 12). In mice, no offspring from presumed totipotent ES cells have been produced by conventional nuclear transfer (12, 13), although offspring were produced when mouse ES cells were chimerized with tetraploid mouse embryos (14).

For domestic animals, morphological identification of putative ES cells has been published (15–18). Pluripotency has been demonstrated for ES cells of swine (17–19), cattle (17, 20, 21), and sheep (19). Injection of newly isolated blastocyst inner cell mass (ICM) cells into other blastocysts has produced chimeric offspring in sheep (22) and cattle (23). Noncultured ICM cells appear to be totipotent as evidenced by blastocyst formation, pregnancies, and offspring after transfer into enucleated oocytes in rabbits (24), sheep (25), and cattle (26).

Cultured cells with ES cell characteristics have been transferred into bovine oocytes initially with the resulting 5-day cultured embryos surviving only to the 8-cell stage (27). More recently, bovine cell lines derived from ICM (20) or morulae (21) have produced pregnancies by nuclear transfer, which fail in the first trimester. Calves have been born from chimeric embryos but the ES cell contribution is as yet unknown. One chimeric fetus was ES cell positive (20). There are no published reports in domestic species that cultured ICM or putative ES cells are totipotent, as evidenced by offspring derived totally from these cells (6, 12, 17, 19–21, 27).

Most attempts to isolate and culture ICM cells have been based on or adapted from the original methods of Evans and coworkers for mice (2, 17). In general, these methods involve separation of blastocyst ICM from trophoblast trophectoderm cells by immunosurgery followed by isolation of cells with stem cell morphological characteristics from ICM cells as they plate down on a fibroblast feeder layer. The putative stem cells are then maintained as a colony on a monolayer of fibroblast cells with differentiation-inhibiting activity, leukemia inhibitory factor, buffalo rat liver (BRL) cells, or BRL conditioned medium added to inhibit differentiation. This system has allowed culture of pluripotent cells that can become embryoid bodies. Aggregated sheets of cells develop cellular beating heart activity. However, only in mice has it allowed demonstration of or maintenance of totipotency of the cultured cells [reviewed by Stewart (6) and Anderson (12)]. It has been suggested that these mouse-derived differentiation-inhibiting agents do not adequately prevent differentiation of stem cells in species other than rodents (12).

We report here the isolation and short-term *in vitro* culture of bovine ICM cells by using a different approach to prevent differentiation. These cells were used in nuclear transfer to produce blastocysts that resulted in the birth of normal calves. This result provides evidence of totipotency of cultured ICM cells in mammalian species other than mouse.

MATERIALS AND METHODS

All embryos used in this experiment were *in vitro* derived from slaughterhouse ovaries and frozen semen by the methods described by Sirard *et al.* (28), Parrish *et al.* (29), and Rosenkrans and First (30). Oocyte maturation was in TC199 containing 10% fetal calf serum (FCS) and 0.5 μg of NIH ovine luteinizing hormone (NIADDK-OLH-25) per ml. Oocytes were fertilized with sperm from any one of five different bulls

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ES cells, embryonic stem cells; SIT, selenium insulin, and transferrin; FCS, fetal calf serum; ICM, inner cell mass.

*To whom reprint requests should be addressed at: University of Wisconsin, 1675 Observatory Drive, Madison, WI 53706.

with sperm concentration and heparin dose adjusted for each bull. At 40–48 hr postfertilization, embryos were manually stripped of all cumulus cells and extraneous sperm by repeated pipetting through a 190-μm fire-polished pipette. Subsequent embryo culture was carried out in a defined medium called CR1aa (30) for 7–8 days at 39°C in 5% CO_2/95% air with high humidity until the embryos had hatched or were fully expanded, after which they were subjected to immunosurgery. Embryos were first washed in 3 ml of TL Hepes with polyvinylpyrrolidone (Sigma; PVP-40) (1 mg/ml) and polyvinyl alcohol (Sigma; p8136) (1 mg/ml) and then washed through four or five CO_2 equilibrated microdrops (50 μl) of CR1aa, polyvinylpyrrolidone, and polyvinyl alcohol under paraffin oil.

Rabbit anti-bovine antibody (1:10 dilution; Sigma; B8270) was added at a 1:10 dilution for a final concentration of 1:100. Embryos were returned to the 39°C incubator for 30 min. The embryos were removed from the incubator and again washed through four or five fresh microdrops of medium. Then guinea pig complement (Sigma; S-1639) was added to the embryos at a 1:10 dilution from a 1:500 diluted stock for a final dilution of 1:5000. While in the presence of complement, the zonae pellucidae were removed by manual pipetting through a non-fire-polished 150-μm pipette tip. The remaining ICMs were washed and then one to three ICMs per 10-μl drop of the medium CR1aa plus SIT (sodium selenite, insulin, and transferrin; Sigma; I 1884) were placed under paraffin oil. Within 5 days, the ICMs started disassociating from a ball of cells into individual free-floating cells. At this time, the ball of cells was mechanically disaggregated by a micromanipulation needle. The medium was changed every 2–3 days by aspiration and replaced with fresh CO_2-equilibrated CR1aa with SIT. The addition of 5% FCS to the medium was beneficial in reducing the "stickiness" of these cells, allowing easier handling during micromanipulation. The ICM cells were maintained as disassociated cells in suspension culture for periods ranging from 1 week to 2 months, depending on the experimental protocol. The culture conditions were derived through a series of experiments comparing the effects of various growth factors, media, and medium supplements on cell maintenance and growth rates. Cell viability was determined by staining with propidium iodide. All embryos used to make cell lines were derived from embryos cultured in the CR1aa/SIT/5% FCS medium.

The cultured ICM cells were used as nuclear donor cells in nuclear transfer. Recipient oocytes were matured *in vitro* (29) and stripped of cumulus 16–18 hr after initiation of oocyte maturation, using hyaluronidase at a concentration of 2 mg/ml and a fire-polished pipette. Oocytes were selected for the presence of polar bodies and returned to maturation medium for another 2–4 hr. Nuclear transfer was begun ≈20 hr after these metaphase II oocytes were placed into culture. Manipulation was performed with a Nikon Diaphot microscope equipped with Hoffman optics and Narishige micromanipulators. Manipulation was done in culture dishes in which microdrops of medium were arranged with each dish containing both 100-μl drops (TL Hepes with Ca^{2+} and Mg^{2+}) in which the oocytes were placed and 20-μl drops (TL Hepes with Ca^{2+} and Mg^{2+} and 20–50% FCS) to one side containing the cultured ICM cells. This was done to prevent the cells from sticking to the oocytes and to prevent mistaking ICM cells with any remaining cumulus cells. Approximately 10 ICM cells were aspirated into the transfer pipette, and then the tips were moved to the drop containing the oocytes. The cells were drawn higher into the pipette to allow space for enucleation of the oocyte. The oocyte was positioned on a holding pipette so that the polar body was toward the transfer tip. A small amount of cytoplasm from the region directly beneath the polar body and the polar body were removed. The transfer tip was retracted from the zona and the cytoplasm was ejected. The tip was reinserted through the same hole and an ICM cell was deposited beneath the zona. The cell was pressed against the plasma membrane, where it stuck firmly between the zona and plasma membrane. Due to the extreme stickiness of the cells, transfer pipettes were changed frequently. Nuclear (ICM cell) transfer was completed by 24 hr postmaturation, and the unfused units were placed in CR1aa medium overnight. All fusions were done with oocytes 42 hr postfollicular removal.

Fusion proved to be a difficult problem because of the disparate sizes of the cells to be fused. The ICM cells ranged in size from 15 to 25 μm, and the enucleated oocytes were ≈140 μm. Except for Table 4, in which recent experiments (bulls F–I) used electrofusion, fusions were with polyethylene glycol (PEG). The fusion protocol used PEG (M_r 1300–1600; Sigma) 1:0.25 g/ml in Ca^{2+}- and Mg^{2+}-free TL Hepes with polyvinyl alcohol (1 mg/ml) for 45 sec followed by a 1:1 dilution in the same medium for 1 min, another 1:1 dilution for 2 min, and then a final 1:1 dilution for 2–3 min. The most reliable PEG was from Boehringer Mannheim (PEG 1500). A 15-min culture in TL Hepes containing 20% FCS allowed membranes to return to their normal appearance. To activate the ooplasm, the embryos were washed through Ca^{2+}- and Mg^{2+}-free TL Hepes and then exposed to 5 mM ionomycin (Calbiochem) in 1 ml of medium for 45 sec. This was followed by another 15-min culture in TL Hepes containing 20% FCS, after which embryos were returned to CR1aa medium for further maturation.

RESULTS

To prevent differentiation, ICM cells were cultured in suspension at a concentration sufficiently low (1000–1500 cells per 10-μl drop) so that cell aggregation and differentiation did not occur. Several differentiation-inhibiting and mitotic factors were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CR1aa plus SIT and either glucose, rifampicin, laminin, or 5% FCS supported mitosis through 2 weeks of culture. Of these, only CR1aa plus SIT plus 5% FCS allowed mitosis and continued proliferation of ICM cells for 4 weeks. ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when cultured in CR1aa plus SIT and 5% FCS with some lines reaching 2000 cells after 2 weeks of culture. These cells have the appearance of mouse ES cells, being small cells with large nuclei, little cytoplasm, and prominent nucleoli (Fig. 1). When

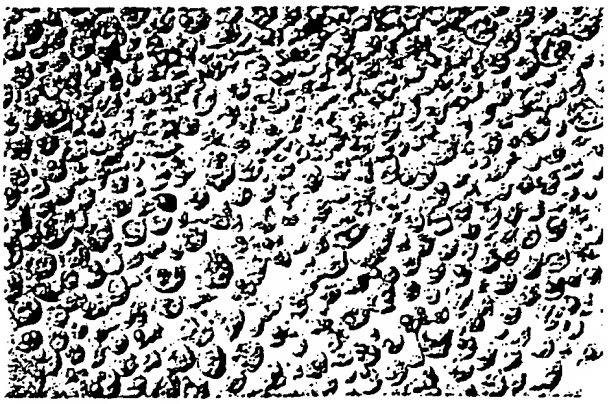


FIG. 1. Bovine ES cells. Cell population in 10-μl microdrops varied from 200 to 2000 cells within 2 weeks of culture. Individual lines were subcultured at 1000–1500 cells per microdrop because embryoid bodies formed when cell population densities exceeded 1000 cells. Note that the nucleus constitutes most of the volume of each cell, the presence of two or three nucleoli per cell, and the large round cells that will soon divide.

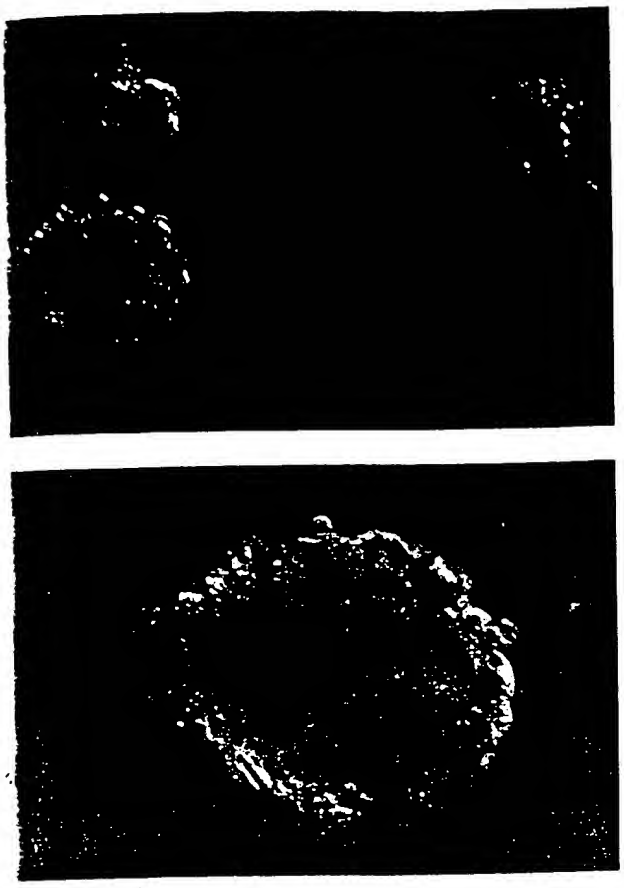


FIG. 2. Embryoid bodies resulting from high cell population density. (*Upper*) Bovine embryoid bodies, two simple and one complex. (*Lower*) Bovine complex embryoid body.

removed from nondifferentiating conditions and allowed to aggregate, the cultured cells formed embryoid bodies (Fig. 2).

Table 1. Effect of number of ICM cells starting a culture on ICM cell survival

No. of ICMs starting cell line	No. of cell lines	
	Started	Surviving at 1 month
1	159	0
3	241	170 (70.54%)

These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

The ability of ICM cells cultured in loose suspension microdrops to establish and maintain a proliferating population of ICM cells appears to be dependent on the number of ICMs used to establish the culture (Table 1). ICM cultures derived from three ICMs from two sires established cultures that proliferated, whereas none of the cultures started from one ICM survived to 10 days and even the surviving cultures had a low proliferation rate, with most dying by 1 month. Occasionally, a single ICM initiated a cell culture as the two shown in Table 2. While one cell line, line six, was maintained for 101 days, most ICM lines derived from pooled embryos lost life and nuclear staining after 3 weeks of culture. Live/dead staining at 3 weeks with calcein AM (live) or ethidium homodimer (dead) showed ≈80% live cells (green) and 20% dead (red) and dying (orange-yellow) cells, whereas, at 5 weeks, nearly 80% of the cells were dead or dying.

One way of accurately determining totipotency of embryonic cells is to fuse the cell in question into an enucleated metaphase II oocyte. We report in Table 2 results from derivation and use of cells from 15 bovine ICM cell lines in nuclear transfer. The cell lines ranged from 6 to 101 days of culture at the time of nuclear transfer. A total of 659 embryos (clones) were made by nuclear transfer. After culture for 7 days *in vitro* in CR1aa and SIT plus 5% FCS, 109 became blastocysts (16.6%); of those cleaving, 25% became blastocysts. Each cell line was derived from the ICM of 3 blastocysts except cell lines 14 and 15, which were each derived from the ICM of a single blastocyst. The efficiency of establishing stem cell cultures from ICM cells and the ability of the oocyte ICM cell fusion product to become a blastocyst

Table 2. Use of loose suspension cultured ICM cells as donors of nuclei in nuclear transfer to produce blastocyts

Cell line	Days PIS to nuclear transfer*	Nuclear transfer clones made†	Cleavage (%)	Blastocysts, n‡	Blastocysts, % of clones§	Blastocysts, % of cleavage
1	35	24	14/24 (58)	6	25	43
2	42	32	20/32 (63)	4	12.5	20
3		33	21/33 (64)	4	12	19
4	17	92	71/92 (77)	19	21	27
5	71	22	18/22 (82)	4	18	22
6	101	22	15/22 (61)	4	18	27
7	6	44	36/44 (82)	9	20	25
7	6	33	2/33 (6)	0	0	0
8	13	57	43/57 (75)	11	19	26
9	20	42	20/42 (48)	6	14	30
10	27	74	61/74 (82)	12	16	20
11	14	23	17/23 (74)	4	17	24
11	14	5	4/5 (80)	2	40	50
12	21	47	40/47 (85)	4	8.5	10
13		28	21/28 (75)	6	21	29
14¶	54	39	21/39 (54)	6	15	29
15¶	61	42	36/42 (86)	8	19	22
	Total	659	460/659 (70)	109	15%	24%

*PIS, postimmunosurgery. Trophoblast cells were removed and culture of ICM cells was initiated.
†Each clone is the product of attempted fusion of an ICM cell with an enucleated oocyte.
‡Number of blastocysts after *in vitro* culture of the clones for 7 days.
§Frequency of clones becoming blastocysts after 7 days of culture.
¶All cell lines were derived from the pooled ICM of 3 blastocysts, except lines 14 and 15, each of which was derived from the ICM of a single blastocyst.

Table 3. Effect of sire on efficiency of stem cell line production and frequency of blastocysts derived fro[illegible]sion of stem cells into enucleated oocytes [nuclear transfer (NT)]

	Sire breed	NT clones made, *n*	NT clones becoming blastocysts	
			n	%
A	Angus	89*	14	15.7
B	Holstein	114*	23	20.2
C	Holstein	272*	42	15.4
D	Brahman	184*	30	16.3
E	Brahman	46†	0	0
F	Longhorn	93†	14	15
G	Holstein	102†	22	22
H	Holstein	60†	6	10
I	Holstein	88†	19	22

*Stem cells were fused into enucleated oocytes by using PEG.
†Stem cells were fused into enucleated oocytes by electrofusion.

appears to be partially dependent on the genetics of the embryo as indicated by differences among sires in the frequency of stem cell line formation and blastocyst formation (Table 3).

Totipotency of cultured ICM cells from five cultured cell lines was determined by transfer into cows of blastocysts derived from ICM cell nuclear transfer (Table 4).

Thirty-four of 42 blastocysts derived from cell lines cultured for 6, 13, 20, 27, or 101 days were transferred into uteri of 27 cows. Thirteen of the cows (49%) became pregnant. At 180 days of gestation, 5 (19%) were still carrying 5 (15%) fetuses with heart beats clearly imaged with ultrasonography. Four of the cows delivered normal calves derived from the cultured ICM cells after gestations of normal length. The birth weights of the calves were 75, 80, and 85 pounds for 3 female calves and 86 pounds for a male calf. The gestations were 279, 280, 280, and 279 days, respectively.

The cell cultures producing offspring were cultures 7, 9, and 10. Cell culture 7 was derived from embryos sired by Holstein bull 9805 and the calf born from this culture was Holstein. Cell culture 9 was derived from embryos sired by Brahman bull 9813 and the two calves born were half Brahman. Cell culture 10 was derived from embryos sired by Longhorn bull 12199 and the calf was half Longhorn. DNA typing by Marijo Kent (31) established that each calf was sired by the sire producing the ICM cells from which the calf was derived. The calves were karyotyped and two half-sister Brangus calves from cell line 9 showed tetraploidy of <10% in some lymphocytes at birth but lost the tetraploid lineage by 1 year of age. Karyotypes of integument fibroblasts were normal.

DISCUSSION

The ICM cell culture system reported here prevents differentiation by culturing cells as a loose suspension with <1500 cells per 10-μl drop. Without cell–cell contact, neither cell aggregation nor monolayer formation occurred.

The results presented in Table 3 show that at least some of the ICM cells retain totipotency after culture. The efficiency of blastocyst production from use of the cultured ICM cells in nuclear transfer (15% or 25% of cleaved) is similar to the efficiency of using morulae cells as the donated nucleus in conventional nuclear transfer (18%; ref. 32).

The frequencies of pregnancies (49%) and 180-day maintained pregnancies (19%) after transfer into cows of embryos derived from cultured ICM cells were also similar to the frequency of pregnancies (30%) or maintained pregnancies reported for conventional bovine nuclear transfer (32, 33). The frequency of transferred blastocysts resulting in born offspring was also similar to bovine nuclear transfer and the calves were from three different cell lines.

Pluripotency has been demonstrated previously for cultured cattle ICM cells (12, 18, 20, 21, 27). Our present research was reported as an abstract in 1993 (34). This work demonstrates totipotency from cultured ICM cells of domestic animals, as evidenced by offspring, and the successful use of cultured cells for nuclear transfer (for review of other species, see ref. 12). The methods presented here allowed establishment of ICM cell lines from ≈70% of the blastocysts attempted, when the line was formed from a pool of 3 ICMs. This is approximately equal to the best efficiencies in the production of mouse ES cell lines (6). In mice, some ES cell lines have been shown to be of abnormal karyotype, particularly after several passages. Whether the tetraploidy of calves from line 9 was due to nuclear transfer or ICM cell culture is unknown. While the efficiency of fusion was acceptable in this study (68%), manufacturers' lots of PEG vary considerably in fusogenic activity. A modified electrofusion procedure was used with success for production of blastocysts from cells derived from sires E, F, G, H, and I in Table 3.

The greatest need will be for cell culture systems that promote much greater mitotic activity than the present system while inhibiting differentiation. The CR1aa/SIT/5% FCS culture medium used for this study is adequate only for short-term culture and represents a mere beginning in identification of an optimal culture system for bovine ICM cells.

With the development of culture systems allowing a high rate of cell multiplication, bovine ES cells derived from ICMs or earlier embryo stages should prove useful in propagation and genetic modification of cattle. The use of ES cells in gene transfer could provide more efficient gene transfer with opportunities to select cells for gene integration or expression before offspring are made and opportunities through homol-

Table 4. Production of calves from blastocysts derived from fusion of cultured ICM cells into enucleated bovine oocytes

Cell line*	Days PIS to NT†	NT clones made, *n*	Blastocysts from NT,‡ *n*	Blastocysts (*n*) transferred into cows (*n*)	Cows pregnant at 42 days gestation, *n*	Blastocysts surviving as fetuses *in utero* at gestation day				Calves born
						56	70	150	180	
7	6	44	9	9 into 6	4	5	4	2	2	1
8	13	57	11	6 into 4	0	—	—	—	—	—
9	20	42	6	6 into 4	3	4	4	4	2	2
10	27	74	12	9 into 9	3	1	1	1	1	1
6	101	22	4	4 into 4	3	0	—	—	—	—
			Total	34 into 27	13 of 27 (49%)	10	9 (27%)	7 (21%)	5 (15%)	4 (12%)

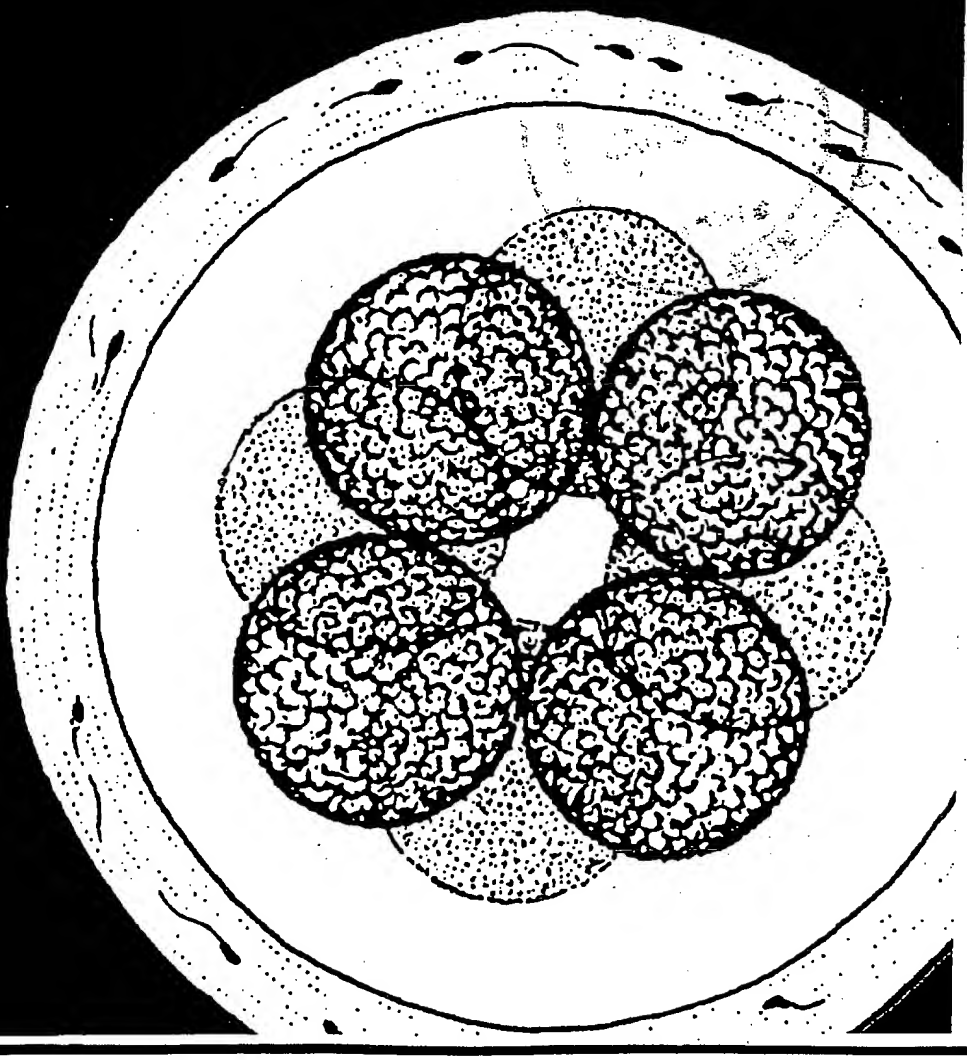
*Each of these cell lines was established from the pooled ICMs of 3 blastocysts.
†PIS, postimmunosurgery and start of ICM cell cultures.
‡Number of blastocysts developed per number of clones made after 7 days of culture.

ogous DNA recombination to be site specific in gene transfer or deletion. Bovine ES cells when used as nuclear donors in nuclear transfer could allow the production of large numbers of clonal offspring from one valuable embryo or from the genetically modified ES cells of one valuable embryo.

This manuscript is dedicated to the memory of Julie Busby. We wish to thank George Rueckert, Sue Ann Hubanks, and Julie Busby for typing this manuscript; Brad Haley for obtaining slaughterhouse material; Valerie Schutzkus for maintaining sterile media stocks and supplies; Dr. Marijo Kent for DNA typing and karyotyping the calves; and Drs. Charlie Rosenkrans and George McNamara for their advice and help. This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and W. R. Grace & Co., Connecticut.

1. First, N. L. & Prather, R. S. (1991) *Differentiation* **48**, 1–8.
2. Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292**, 154–156.
3. Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9065–9069.
4. Lovell-Badge, R. H., Bygrave, A., Bradley, A., Robertson, E. J., Tilly, R. & Cheath, K. S. E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2803–2807.
5. Joyner, A. L. (1991) *Bioassays* **13**, 649–658.
6. Stewart, C. L. (1991) in *Animal Applications of Research in Mammalian Development*, eds. Pedersen, R. A., McLaren, A. & First, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 267–283.
7. Hooper, M. L., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326**, 292–295.
8. Koller, B. H., Hagemann, L. J., Doetschman, T., Hagerman, J. R., Huang, S., Williams, P. J., First, N. L., Maeda, N. & Smithies, O. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8927–8931.
9. Capecchi, M. R. (1989) *Science* **244**, 1288–1292.
10. Stanton, B. R., Reid, S. W. & Parada, L. F. (1990) *Mol. Cell. Biol.* **10**, 6755–6758.
11. Yagi, T., Ikawa, Y., Yoshida, K., Shigetani, Y., Takedo, N., Mabuchi, I., Yamamoto, T. & Aizawa, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9918–9922.
12. Anderson, G. B. (1992) *Anim. Biotechnol.* **3**, 165–175.
13. Modlinski, J. A., Gerhauser, D., Lioi, B., Winking, H. & Illmensee, K. (1990) *Development* **108**, 337–348.
14. Nagy, A., Ro[illegible], J., Nagy, R., Abramov-Newerly, W. & Roder, J. C. (1[illegible] *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428.
15. Piedrahita, J. A., Anderson, G. B. & BonDurant, R. H. (1990) *Theriogenology* **34**, 879–901.
16. Piedrahita, J. A., Anderson, G. B. & BonDurant, R. H. (1990) *Theriogenology* **34**, 865–877.
17. Evans, M. J., Notarianni, E., Laurie, S. & Moor, R. M. (1990) *Theriogenology* **33**, 125–128.
18. Notarianni, E., Laurie, S., Moor, R. M. & Evans, M. J. (1990) *J. Reprod. Fertil. Suppl.* **41**, 51–56.
19. Notarianni, E., Galli, C., Laurie, S., Moor, R. M. & Evans, M. J. (1991) *J. Reprod. Fertil. Suppl.* **43**, 255–260.
20. Stice, S., Strelchenko, N., Betthauser, J., Scott, B., Jurgella, G., Jackson, J., David, V., Keefer, C. & Matthews, L. (1984) *Theriogenology* **41**, 301 (abstr.).
21. Strelchenko, N. & Stice, S. (1994) *Theriogenology* **41**, 304 (abstr.).
22. Butler, J. E., Anderson, G. B., BonDurant, R. H., Pashen, R. L. & Penedo, M. C. T. (1987) *J. Anim. Sci.* **65**, 317–324.
23. Summers, P. M., Shelton, J. M. & Bell, K. (1983) *Anim. Reprod. Sci.* **6**, 91–102.
24. Collas, P. & Robl, J. (1991) *Biol. Reprod.* **43**, 877–884.
25. Smith, L. C. & Wilmut, T. (1989) *Biol. Reprod.* **40**, 1027–1035.
26. Keefer, C. L., Koppang, R., Paprocki, A. M., Galueke, P., Stice, S., Maki-Laurila, M. & Mathews, L. (1993) *Theriogenology* **39**, 242 (abstr.).
27. Saito, S., Streichenko, N. & Niemann, H. (1992) *Roux's Arch. Dev. Biol.* **201**, 134–141.
28. Sirard, M. A., Parrish, J. J., Ware, C. B., Leibfried-Rutledge, M. L. & First, N. L. (1988) *Biol. Reprod.* **39**, 546–552.
29. Parrish, J. J., Susko-Parrish, J., Winer, M. A. & First, N. L. (1988) *Biol. Reprod.* **38**, 1171–1180.
30. Rosenkrans, C. F. & First, N. L. (1993) *J. Anim. Sci.* **72**, 434–437.
31. First, N. L., Sims, M. M., Park, S. P. & Kent-First, M. J. (1994) *J. Reprod. Fertil. Suppl.*, in press.
32. Barnes, F. L., Westhusin, M. E. & Looney, C. R. (1990) in *4th World Congress on Genetics Applied to Livestock Production* (Edinburgh Univ., Edinburgh, Scotland), Vol. 16, pp. 323–333.
33. Bondioli, K. R., Westhusin, M. E. & Looney, C. R. (1990) *Theriogenology* **33**, 165–174.
34. Sims, M. M. & First, N. L. (1993) *Theriogenology* **39**, 313 (abstr.).

Reproduction, Fertility and Development



3613

Reprod. Fertil. Dev., 1990, 2, 619–22

In vitro Embryo Culture in the Production of Identical Merino Lambs by Nuclear Transplantation

K. J. McLaughlin[A], *L. Davies*[B] *and R. F. Seamark*[A]

[A] Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA 5000, Australia.
[B] Australian Sheep Artificial Breeders, Nairne, SA 5252, Australia.

Abstract

This study examined the viability of embryos developed *in vitro* from 8- to 16-cell stage blastomeres fused with enucleated oocytes. Of 209 blastomeres recovered and subjected to manipulation and electrofusion procedures, 190 (91%) fused successfully, with 86 (45%) of those undergoing cleavage up to the 4- to 16-cell stage when cultured for 66 h in a synthetic oviduct fluid medium. The viability of the embryos was examined by transferring them to recipient ewes and determining the ewes' pregnancy status by ultrasound on Day 45. Of 86 embryos transferred, 14 developed to fetuses in 8 of the 36 recipients, including four sets of identical twins and one set of quads. In contrast, with uncultured and unmanipulated embryos, 15 fetuses developed from 19 embryos transferred at a similar stage of development. The viability of embryos derived from manipulated zygotes cultured *in vitro* was comparable to that previously reported for studies employing *in vivo* culture, indicating the potential of *in vitro* culture systems based on a simple medium for nuclear-transplantation embryos.

Introduction

Production of identical sheep is possible through the transplantation of cleavage stage nuclei into enucleated oocytes (Willadsen 1986; Smith and Wilmut 1989). To minimize losses of cloned embryos due to damage to the zona pellucida from the manipulation procedures, zygotes have been temporarily transferred to ovine oviducts encased in agar gel capsules and recovered at the morula–blastocyst cell stage for transfer to recipient ewes (Willadsen 1986). Recently, several procedures have been developed that allow *in vitro* culture of ovine zygotes for up to 3 days, one such procedure being the use of a simple medium based on the composition of sheep oviduct fluid (Walker *et al.* 1988). This paper examines the use of *in vitro* culture as a means of both simplifying procedures for handling zygotes created by nuclear transplantation prior to transfer and allowing detailed study of the early developmental stages.

Materials and Methods

Oocyte and Embryo Collection

Superovulation was induced in New South Wales Merino ewes with 12-day progestagen pessaries (60 mg medroxyprogesterone acetate each; Upjohn Pty Ltd, Sydney, New South Wales) and 22 mg per ewe of follicle-stimulating hormone (Heriot Agvet Pty Ltd, Melbourne, Victoria). Synthetic gonadotrophin-releasing hormone (50 mg i.m. per ewe; Intervet Australia Pty Ltd, Artarmon, New South Wales) was administered 30 h after pessary removal. Ewes were inseminated via a laparoscope (Walker *et al.* 1984) with a minimum of 20×10^6 motile fresh spermatozoa per uterine horn 48 h after pessary removal.

1031-3613/90/060619$03.00

Oocytes and embryos were collected by mid-ventral laparotomy approximately 12–17 h and 60 h, respectively, after the expected median time of ovulation. Oviducts were flushed with 10 mL Dulbecco's phosphate-buffered saline (Flow Laboratories, North Ryde, New South Wales) containing 10% heat-inactivated sheep serum. Oocytes and embryos were recovered from the flushings within 5 min of collection.

Culture and Transport

Embryos were cultured in synthetic oviduct fluid medium (SOFM) according to the formulation of Tervit *et al.* (1972), but with 20% heat-inactivated human serum (HS) in lieu of bovine serum albumin (Walker *et al.* 1986). Transportation of embryos was in tubes containing SOFM–HS supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'2-ethanesulfonic acid). The transport medium was pre-equilibrated in an atmosphere of 5% CO_2 in air at 37°C, and transport between the collection site and the laboratory was accomplished at 37°C. The time between collection and initiation of manipulation was 2–4 h. Embryos were cultured after manipulation in 10 μL drops of SOFM–HS under paraffin oil in 35 mm plastic dishes in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The microdrop culture was pre-equilibrated in the culture environment 24 h prior to the addition of embryos. Incubation was maintained at 38·5°C and 95% relative humidity.

Manipulation Procedures

Manipulations were carried out at room temperature (25°C). The manipulation medium for donor-nuclei embryos and oocytes was SOFM supplemented with 25 mM HEPES, 5 mM $NaHCO_3$, 7·5 mg mL^{-1} cytochalasin B (CB; Sigma. St Louis, Missouri), and 10% heat-inactivated sheep serum. Donor-nuclei embryos and oocytes were placed in SOFM–CB for 15 min prior to manipulation. Isolation of donor-nuclei blastomeres, enucleation of oocytes, and blastomere transfer were essentially as described by Prather *et al.* (1987).

Electrofusion was perfomed with an apparatus similar to that described by Kubiak and Tarkowski (1986) but modified by the addition of an a.c. pulse generator (Topward TFG-8104, Taiwan). Electrical output was monitored with an oscilloscope (Goodwill GOS-522B, Sydney, New South Wales). For fusion, manipulated embryos were placed in a solution of 0·3 M mannitol, 0·1 M $MgSO_4$, and 0·05 mM $CaCl_2$ (Willadsen 1986) for 10 min and positioned between platinum electrodes placed 200 mm apart. Manipulated embryos were then aligned in an a.c. field of 3 V, 500 kHz, followed by a 13 V d.c. pulse of 100 ms duration, followed by a reduction of the a.c. field strength to 0 V over 15 s after the d.c. pulse. The embryos were then cultured in SOFM–CB for 1 h to increase development (Smith and Wilmut 1989), and fusion rates were recorded before the embryos were placed in the SOFM–HS microdrop culture. Embryos were observed for cleavage every 24 h after fusion. To determine the number of viable embryos remaining after selection on the basis of morphological criteria (see below), the remaining embryos from the last three trials were cultured a further 56 h and the number of nuclei determined by staining with Hoechst 33342 (Sigma, St Louis, Missouri) (Pursel *et al.* 1985) and examination with fluorescence microscopy (200×).

Embryo Transfers

Manipulated embryos were selected from culture at 66 post-fusion on the basis of morphological appearance and cleavage regularity during culture. One to five embryos were transferred to individual ewes according to recipient availability, condition of embryos, and number of clones derived from each donor embryo. Embryos collected at the same stage as donor embryos were also transferred to synchronized recipients within 2–3 h of collection to test the survival of uncultured and unmanipulated embryos. Recipient animals were tested for pregnancy by ultrasound on Day 45 to determine the number of fetuses *in utero*.

Results

A total of 31 donor embryos from seven ewes were selected for nuclear transfer, yielding 209 successfully manipulated blastomeres. Of these, 190 (91%) fused, and 86 (45%) of those underwent cleavage up to the 4- to 16-cell stage during 66 h of culture and were subsequently transferred to 36 recipient ewes. Nuclei counts in embryos not selected for transfer (84) indicated that 40 (48%) had undergone cleavage to various stages (Table 1),

with 12 (14%) of those forming blastocysts following a further 54 h of culture. The cell numbers of blastocysts forming in culture ranged from 6 to 32.

At Day 45, 8 (22%) of the 36 recipients receiving the manipulated and cultured embryos were pregnant (Table 2), and 14 fetuses (16% of the 86 embryos transferred) were detected by ultrasound. Transfer of untreated control embryos (19) to recipients (7) resulted in all animals becoming pregnant with a total of 15 fetuses (79%) *in utero*. Amongst the pregnancies from manipulated and cultured embryos, there were six sets of twins and two singletons. Each set of twins was derived from a single embryo, with two of the sets originating from the same embryo.

Table 1. *In vitro* development of untransferred embryos at 120 h post-fusion

Embryos cultured	2–16 cells	>16 cells	Blastocysts	Nuclei per blastocyst[A]
84	33 (39%)	7 (8%)	12 (14%)	14 ± 7·1 (6–32)

[A] Mean ± s.d.; range in parentheses.

Table 2. Development of manipulated embryos cultured for 66 h post-fusion and of control embryos of similar age

	Embryos transferred[A]	Pregnancies	Fetuses
Manipulated	86 (36)	8 (22%)	14 (16%)[B]
Control	19 (7)	7 (100%)	15 (79%)

[A] Number of recipients in parentheses.
[B] Significantly different from control group (χ^2 analysis; $P < 0{\cdot}01$).

Discussion

The results indicate that the *in vitro* culture system developed by Walker *et al.* (1988) can be used to support the early development of nuclear-transplantation embryos. The pregnancy rate achieved (22%) is similar to that obtained with *in vivo* culture of nuclear-transfer embryos from sheep (17%; Smith and Wilmut 1989) and cattle (22·5%; Bondioli *et al.* 1990), with the major loss of embryos occurring early in pregnancy at either implantation or shortly thereafter. Unmanipulated ovine embryos cultured in the same system for a similar period *in vitro* and transferred produce high numbers of elongated conceptuses (94%) at Day 14 (Walker *et al.* 1988). Thus, the losses experienced with micromanipulated embryos more likely relate to the manipulation procedures than to deficiencies in the culture system or to the inability of the donor nuclei to be reprogrammed sufficiently to support development.

Overall embryo survival after transfer was 16%, with 70% of embryos forming fetuses in recipients that became pregnant and with a higher number of twin than singleton pregnancies (6:2). In mouse embryos, variation in the cell cycle stage of donor nuclei can influence development following transfer to the enucleated zygote (Smith *et al.* 1988), and this may account for the large variation seen in the rate of development of manipulated sheep embryos in culture. This variation may cause losses due to asynchrony between embryos and recipients (Rowson *et al.* 1966).

The morphological criteria used to screen embryos after 66 h of *in vitro* culture identified the majority of the embryos as suitable for transfer, with only 7 (8%) of the 84 remaining

embryos undergoing further, albeit impaired, development as assessed by the low cell numbers. There are no comparable reports on the survival of ovine embryos cultured *in vitro* following nuclear transfer, but bovine nuclear-transfer embryos have been maintained to the blastocyst stage in SOFM-HS (Seamark, unpublished observations) and in co-culture (Bondioli *et al.* 1990), with subsequent pregnancies. Further extension of the culture period for the manipulated embryos prior to transfer was not attempted as experience with unmanipulated embryos (Gandolfi and Moor 1987; Walker *et al.* 1988) indicates that this would probably result in a further reduction in the pregnancy rate.

The present study indicates that the simple *in vitro* culture system described may provide a viable alternative to the *in vivo* culture systems that have previously been employed for short-term culture of manipulated zygotes. If, as the present data suggest, the *in vitro* system is as efficacious as the *in vivo* systems, it can be recommended as the method of choice since it is not only simpler to manage but also has the advantage of permitting direct observation of embryos, thus enabling experimentation aimed at improving understanding of the factors that limit the early critical stages of embryo development. Further studies aimed at providing a direct comparison of the efficacies of the *in vitro* and *in vivo* systems are warranted.

Acknowledgments

We thank Leonie Hicks and the staff of Australian Sheep Artificial Breeders for technical assistance. This work was financially supported in part by Emtech. All experiments were approved and carried out under the guidelines of the University of Adelaide Animal Ethics Committee.

References

Bondioli, K. R., Westhusin, M. E., and Looney, C. R. (1990). Production of identical bovine offspring by nuclear transfer. *Theriogenology* **33**, 165–74.

Gandolfi, F., and Moor, R. M. (1987). Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J. Reprod. Fertil.* **81**, 23–8.

Kubiak, J. Z., and Tarkowski, A. J. (1986). Electrofusion of mouse blastomeres. *Exp. Cell Res.* **157**, 561–6.

Prather, R. S., Barnes, F. L., Sims, M. M., Robl, J. M., Eyestone, W. H., and First, N. L. (1987). Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol. Reprod.* **37**, 859–66.

Pursel, V. G., Wall, R. J., Rexroad, C. E., Jr, Hammer, R. E., and Brinster, R. L. (1985). A rapid whole mount staining procedure for nuclei of mammalian embryos. *Theriogenology* **24**, 687–91.

Rowson, L. E. A., Lawson, R. A. S., and Moor, R. M. (1971). Production of twins in cattle by egg transfer. *J. Reprod. Fertil.* **25**, 261–8.

Smith, L. C., and Wilmut, I. (1989). Influence of nuclear and cytoplasmic activity on the development *in vivo* of sheep embryos after nuclear transplantation. *Biol. Reprod.* **40**, 1027–35.

Smith, L. C., Wilmut, I., and Hunter, R. H. F. (1988). Influence of cell cycle stage at nuclear transplantation on the development *in vitro* of mouse embryos. *J. Reprod. Fertil.* **84**, 619–24.

Tervit, H. R., Whittingham, D. G., and Rowson, L. E. A. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil.* **30**, 493–7.

Walker, S. K., Smith, D. H., Little, D. L., Warnes, G. M., Quinn, P., and Seamark, R. F. (1984). Artificial insemination and transfer of embryos by laparoscopy. In 'Reproduction in Sheep'. (Eds D. R. Lindsay and D. T. Pearce.) pp. 306–9. (Australian Academy of Science: Canberra.)

Walker, S. K., Quinn, P., Ashman, R. J., Smith, D. H., and Seamark, R. F. (1986). Protein supplementation for the culture of one-cell embryos in sheep. *Proc. Aust. Soc. Reprod. Biol.* **18**, 9.

Walker, S. K., Seamark, R. F., Quinn, P., Warnes, G. M., Ashman, R. J., Smith, D. H., and Ancell, P. (1988). Culture of pronuclear embryos of sheep in a simple medium. *Proc. 11th Int. Congr. Anim. Reprod. A.I.* **4**, 483–5.

Willadsen, S. M. (1986). Nuclear transplantation in sheep embryos. *Nature (Lond.)* **320**, 63–5.

Manuscript received 5 April 1990, revised and accepted 27 July 1990

BIOLOGY OF REPRODUCTION 41, 414–418 (1989)

Nuclear Transplantation in Early Pig Embryos[1]

RANDALL S. PRATHER,[2] MICHELLE M. SIMS, and NEAL L. FIRST[3]

Department of Meat and Animal Science
College of Agricultural and Life Sciences
University of Wisconsin-Madison
Madison, Wisconsin 53706

ABSTRACT

Nuclear transfer was evaluated in early porcine embryos. Pronuclear stage embryos were centrifuged, treated with cytoskeletal inhibitors, and subsequently enucleated. Pronuclei containing karyoplasts were placed in the perivitelline space of the enucleated zygote and fused to the enucleated zygote with electrofusion. The resulting pronuclear exchange embryos were either monitored for cleavage in vitro (9/13 cleaved and contained 2 nuclei after 24 h, 69%) or for in vivo development. In vivo development after 3 days resulted in 14/15 (93%) of the embryos transferred cleaving to the ≥4-cell stage and after 7 days 6/16 (38%) reaching the expanded blastocyst stage. A total of 56 pronuclear exchange embryos were allowed to go to term, and 7 piglets were born.

A similar manipulation procedure was used to transfer 2-, 4- or 8-cell nuclei to enucleated, activated meiotic metaphase II oocytes. Enucleation was effective in 74% (36/49) of the contemporary oocytes. Activation was successful in 81% (37/46) of nonmanipulated but pulsed oocytes versus 13% (4/31) of control oocytes ($p<0.01$). After 6 days in vivo, 9% (1/11) of the 2-cell nuclei, 8% (7/83) of the 4-cell nuclei, and 19% (11/57) of the 8-cell nuclei transferred to enucleated, activated meiotic metaphase II oocytes resulted in development to the compact morula or blastocyst stage ($p<0.01$). A total of 88 nuclear transfer embryos were transferred to recipient gilts for continued development. A single piglet was born after the transfer of a 4-cell nucleus to an enucleated, activated metaphase II oocyte and subsequent in vivo development. Therefore 4-cell nuclei are capable of directing development to term after transfer to an enucleated, activated meiotic metaphase II oocyte.

INTRODUCTION

The transfer of nuclei between the cells of early mammalian embryos has been accomplished in a variety of species, including mice (McGrath and Solter, 1983a; Robl et al., 1986), sheep (Willadsen, 1986), cattle (Prather et al., 1987; Robl et al., 1987), and rabbits (Stice and Robl, 1988), and has been described briefly for pig embryos (Robl and First, 1985). Nuclear transfer studies have served to describe nuclear versus cytoplasmic inheritance (McGrath and Solter, 1983b, 1984a; Mann, 1986; Robl et al., 1988) and imprinting during gametogenesis (Surani et al., 1986; Barra and Renard, 1988), as well as to determine the extent of nuclear differentiation in early development (Willadsen, 1986; Prather et al., 1987; Stice and Robl, 1988). Interestingly, the results obtained to date for all mammalian embryos other than mouse embryos suggest that nuclei from early cleavage stages can be reprogrammed to behave as 1-cell embryos if transferred to enucleated, activated meiotic metaphase II oocytes (reviewed by Prather and First, 1989).

Since all mammalian nuclei do not respond to nuclear transfer as mouse nuclei do, it is important to determine the affects of nuclear transfer in mammals other than the mouse. Here we report data showing that pronuclei can be exchanged successfully between porcine zygotes and result in normal offspring, and that some cleavage-stage nuclei are capable of directing complete development after transfer to an enucleated, activated meiotic metaphase II oocyte.

MATERIALS AND METHODS

Source of Embryos

Crossbred (Yorkshire × Landrace) gilts were monitored twice daily for signs of estrus. Oocytes were collected from nonmated animals 36 or 48 h after first-detected estrus, and pronuclear, 2-cell, 4-cell, and

Accepted May 2, 1989.
Received March 14, 1989.
[1]This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture under agreement No. 88-37240-3755. Some of these data have been presented previously in abstract form: see Prather et al. (1988, 1989a).
[2]Present address: Department of Animal Sciences, University of Missouri-Columbia, Columbia, MO 65211.
[3]Reprint requests.

8-cell embryos were collected from mated animals at 48–72 h after the onset of estrus. Embryos were collected by flushing the oviducts and/or uteri with N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered Tyrode's media (HbT; Bavister et al., 1983) supplemented with bovine serum albumin (3 mg/ml, Sigma, St. Louis, MO) and gentamicin sulfate (50 μg/ml; Sigma).

Macromanipulation

Micromanipulation of pronuclear embryos was completed as described for the bovine by Robl et al. (1987). Briefly, pronuclear stage embryos were centrifuged at 15,000 × *g* for 3 min to allow visualization of the pronuclei (Wall et al., 1985; Robl et al., 1987). Centrifuged pronuclear embryos were placed in HbT containing 7.5 μg/ml cytochalasin B (Sigma) and 0.1 μg/ml demecolcine (Sigma) for 5 min prior to macromanipulation. A single embryo was held in place by a holding pipette attached to a Narshigie micromanipulator. Next a 30–33 μm (external diameter, O.D.), beveled, sharpened, glass pipette, attached to another Narshigie micromanipulator, was inserted into the embryo and moved adjacent to the pronuclei. The pronuclei were then aspirated into the pipette, and the pipette was removed. This permitted the removal of the pronuclei within a membrane bound karyoplast. This karyoplast was transferred to an enucleated zygote. The membranes were then fused as described below.

Nuclear transfer procedures were carried out as described by Prather et al. (1987) in cows. Briefly, after embryos and oocytes were exposed to HbT containing cytochalasin B, but not demecolcine, oocytes were prepared by aspirating the first polar body and cytoplasm directly underneath (presumably containing the metaphase chromosomes) with a 30–33 μm (O.D.) pipette. A karyoplast from a 2-cell, 4-cell, or 8-cell stage embryo was then aspirated into the transfer pipette and expelled into the perivitelline space of the enucleated oocyte. Electrically induced membrane fusion was conducted as described below. Some oocytes were enucleated, stained, and evaluated for the presence of meiotic metaphase II chromosomes. Other oocytes were not manipulated but were exposed to similar conditions and then to the electrofusion pulse, or were sham-pulsed and cultured 24 h and examined for the presence of a single pronucleus.

Membrane Fusion

Membranes were fused by electrofusion (Prather et al., 1987; Robl et al., 1987). The nuclear transfer embryos were placed between two platinum electrodes 1 mm apart in a solution of 0.3 M mannitol (Sigma). The embryos were manually oriented so that the fusion plane was parallel to the electrodes, exposed to a 5 V AC (1000 KHz, 20% duty on) field for 5–10 s, and a 30-μs, 120 V/mm (DC) pulse was then applied. The AC field was programmed to decrease to 0 V over 5 s following the DC pulse. Power was provided by a Zimmermann Cell Fusion™ instrument (Model Z1000, GCA/Precision Scientific Group, Chicago, IL).

Culture

In vitro culture was conducted in 50-μl drops of TCM 199 supplemented with 10% heat-treated fetal bovine serum (Gibco, Grand Island, NY) under paraffin oil at 39°C under an atmosphere of 100% humidity and 5% CO_2 in air. Nuclear transfer embryos that had fused and were destined for in vivo culture were transferred to the oviducts of synchronized recipient gilts. In some gilts, pregnancy was terminated at slaughter and embryos were recovered by flushing the oviducts or uteri with HbT. These embryos were evaluated by phase-contrast microscopy or after fixation and staining with aceto-orcein and phase-contrast microscopy. Some of the blastocyst-stage embryos were transferred to the uteri of synchronized recipient gilts. In some cases, the recipient gilts were bred to a Hampshire boar to aid in maintaining the pregnancy by providing enough conceptuses while providing a color marking to identify offspring. Estrus was subsequently monitored twice daily with the aid of a boar.

Data were analyzed by chi-square (Snedecor and Cochran, 1980).

RESULTS

Pronuclear Exchange

Pronuclear exchanges were conducted to determine if the procedures developed for nuclear transfer were detrimental to continued development. Electrofusion was successful in 76% (89/117) of the manipulated zygotes. After 24 h of in vitro cultures, 9/13 (69%) of the pronuclear exchange embryos had cleaved and had 2 nuclei. After 3 days in vivo, 14/15 embryos contained 4 or more cells; these 15 embryos were subsequently transferred to another recipient gilt. Thirty-eight percent (6/16) of the pronuclear exchange embryos cultured in vivo for 7 days were recovered as expanded blastocysts:

four of these embryos were subsequently transferred to another recipient gilt. This resulted in a total of 56 pronuclear exchange embryos transferred to 6 recipient gilts that were allowed to continue to term (Table 1). One of these animals returned to estrus on Day 16, one on Day 50, and one on Day 93. The remaining 3 gilts farrowed a total of 32 pigs, 7 of which were derived from the transfer of 35 pronuclear exchange embryos.

Cloning

An examination of the steps involved with the methods for cloning revealed that 74% (36/49) of contemporary meiotic metaphase II oocytes were in fact enucleated. The pulse required for fusion activated 81% (37/46; as judged by the presence of a single pronucleus after 24 h of in vitro culture) of meiotic metaphase II oocytes that were not manipulated, whereas sham-pulsed oocytes activated 13% (4/31) of the time ($p<0.01$). Electrofusion rates were not significantly different between donor cell stages (2-cell, 59/77, 77%; 4-cell, 115/138, 83%; 8-cell, 71/83, 86%; $p>0.25$; this is a retrospective analysis).

In vitro development showed that 55% (11/20) of the 2- and 4-cell nuclei transferred, participated in a cleavage division (2-cell, 7/15; 4-cell, 4/5) after 24 h. After 6 days in vivo, 9% (1/11) of 2-cell donor nuclei, 8% (7/83) of 4-cell donor nuclei, and 19% (11/57) of the 8-cell donor nuclei ($p<0.01$) transferred to an enucleated, activated meiotic metaphase II oocyte had developed to the compact morula or expanded blastocyst stage (4 of the 4-cell donor nuclei-derived blastocyst-stage embryos were subsequently transferred to another recipient gilt; the other three were not transferred because a synchronized recipient was not available). A total of 42 nuclear transfer embryos were transferred to 4 nonbred recipients and allowed to continue pregnancy. One had a normal cycle, one had a 28-day cycle, and 2 had cycles of 52 days or more (Table 2). A total of 46 nuclear transfer embryos were transferred to 5 bred recipients. One 4-cell nuclear transfer piglet and forty-eight control piglets were born from these 5 gilts (Table 2).

TABLE 1. Establishment and maintenance of pregnancy after the transfer of pronuclear exchange embryos to recipient gilts.

Number transferred	Recipient	Pregnancy result
9	25[a]	estrus detected Day 16
8	1–4[a]	estrus detected Day 50
4[b]	17[c]	estrus detected Day 93
8	32[a]	4 pronuclear exchange pigs born 10 control pigs born
12	51[a]	2 pronuclear exchange pigs born 7 control pigs born
15[b]	7–7[a]	1 pronuclear exchange pig born 8 control pigs born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

TABLE 2. Pregnancy establishment and maintenance after the transfer of 2-, 4- or 8-cell nuclear transfer embryos to recipient gilts.

Number transferred	Donor cell stage	Recipient	Pregnancy result
10	2	25	extended cycle, 28 days
12	2	6809521	extended cycle, 52 days
11	2	509[a]	13 control piglets born
7[b]	4	55[a]	6 control piglets born 1 nuclear transfer pig born
4	4	234[c]	normal cycle
16	4	7352	extended cycle, 72 days
7	4	230[a]	11 control piglets born
17	8	46[a]	10 control piglets born
4	8	7372[a]	8 control piglets born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

DISCUSSION

The results presented in this paper show that pig zygotes can tolerate the conditions necessary for pronuclear exchange and continue development to term. The results further show that oocytes in meiotic metaphase II can be enucleated, activated, fused with a 4-cell karyoplast, and subsequently direct development to the blastocyst stage and to term.

Pronuclear exchange has been used to study the nuclear versus cytoplasmic inheritance of cell surface antigens (SSEA-3; McGrath and Solter, 1983b), lethal mutations (T^{hp}: McGrath and Solter, 1984a), imprinting (Surani et al., 1986), and control of early development (Robl et al., 1988). These studies were all conducted in mouse embryos, but the application of the techniques developed by McGrath and Solter (1983a) to the cow embryo (Robl et al., 1987) and now to the pig embryo permit similar investigations in these domestic species.

Nuclear transfer for cloning has been successful in embryos from amphibians (reviewed by Gurdon, 1986;

DiBerardino, 1987; Prather, 1989) as well as in embryos from sheep (Willadsen, 1986), cattle (Prather et al., 1987), rabbits (Stice and Robl, 1988), and mice (Tsunoda and Shioda, 1988), although it is not clear if any nuclear reprogramming has resulted from this nuclear transfer (McGrath and Solter, 1984b; Barnes et al., 1987; Howlett et al., 1987). A major developmental difference between these animal embryos is the timing of the transition from maternal control of development (relying upon maternally stored RNA) to zygotic control of development (relying upon zygotically produced RNA). The major transition appears to occur at the mid-blastula stage for *Xenopus* embryos (~4000-cell stage; Newport and Kirschner, 1982), 8- to 16-cell stage for sheep embryos (Calarco and McLaren, 1976; Crosby et al., 1988), 8- to 16-cell stage for cow embryos (Camous et al., 1986; Barnes, 1988; King et al., 1988) and 2-cell stage for mouse embryos (Bolton et al., 1984). Why nuclear transfer for cloning is successful in species other than the mouse is not known, but it is interesting to note that the transition to zygotic control of development for the mouse occurs at an earlier time than in the other species. Pig embryos appear to fall between cow, sheep, and mouse embryos and make a transition by the 8-cell stage (White et al., 1987; Prather et al., 1989b); they may begin producing rRNA by the 4-cell stage, since this is the time at which nucleoli are first seen to begin reticulating (Norberg, 1970).

In vivo development to the morula/blastocyst stage was lower for the 2-cell and 4-cell donor nuclei versus the 8-cell donor nuclei ($p<0.01$). However, because of the limited number of replications, high degree of variability between replications, and the fact that these are retrospective data, no firm conclusions should be inferred.

The fusion percentage was not significantly different for the different donor cell stages and resembles that found for the 2-cell to 8-cell stage bovine blastomeres (Prather et al., 1987).

A major complicating factor in conducting these experiments is the lack of tight control over the stage of the cell cycle at which the embryos are collected and manipulated. This should not present a problem for the pronuclear exchanges, because the zygotes should be close to the same stage of the cell cycle–probably all in S. However, for cloning, a single embryo collection may contain cells of 2 different cleavage stages, "late" 4-cell and "early" 8-cell embryos, e.g. 4-cell embryos and 5- to 8-cell embryos. In this paper, these late 4-cell donor nuclei were pooled with early 4-cell donor nuclei, e.g. a single collection containing 2-cell and 4-cell stage embryos. Although differences in stage of the cell cycle for nuclear transfer are not important for early amphibian nuclei (Ellinger, 1978), such effects of stage of the cell cycle are important for more slowly dividing cells (Von Beroldington, 1981), but this has not been evaluated in mammals. Future studies of ours will attempt to evaluate the differences between early and late cell stage donor nuclei.

REFERENCES

Barnes FL, 1988. Characterization of the onset of embryonic control and early development in the bovine embryos. Ph.D. Dissertation. University of Wisconsin-Madison

Barnes FL, Robl JM, First NL, 1987. Nuclear transplantation in mouse embryos: assessment of nuclear function. Biol Reprod 36:1267–74

Barra J, Renard J-P, 1988. Diploid mouse embryos constructed at the late 2-cell stage from haploid parthenotes and androgenotes can develop to term. Development 102:773–79

Bavister BD, Leibfried ML, Lieberman G, 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol Reprod 28:235–47

Bolton VN, Oades PJ, Johnson MH, 1984. The relationship between cleavage, DNA replication and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol 79:139–63

Calarco PG, McLaren A, 1976. Ultrastructural observations of preimplantation stages of the sheep. J Embryol Exp Morphol 36:609–22

Camous S, Kopecny V, Flechon J-E, 1986. Autoradiographic detection of the earliest stage of ^{3}H-uridine incorporation into the cow embryo. Biol Cell 58:195–200

Crosby IM, Gandolfi F, Moor RM, 1988. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 82:769–75

DiBerardino MA, 1987. Genomic potential of differentiated cells analyzed by nuclear transplantation. Am Zool 27:623–44

Ellinger MS, 1978. The cell cycle and transplantation of blastula nuclei in *Bombiana orientalis*. Dev Biol 65:81–89

Gurdon JB, 1986. Nuclear transplantation in eggs and oocytes. J Cell Sci 4 (Suppl):287–318

Howlett SK, Barton SC, Surani MA, 1987. Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. Development 101: 915–23

King WA, Nair A, Chartrain I, Betteridge KJ, Guay P, 1988. Nucleolus organizer regions and nucleoli in preattachment bovine embryos. J Reprod Fertil 82:87–95

Mann JR, 1986. DDK egg-foreign sperm incompatibility in mice is not between the pronuclei. J Reprod Fertil 76:779–81

McGrath J, Solter D, 1983a. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220:1300–02

McGrath J, Solter D, 1983b. Nuclear transplantation in the mouse embryos. J Exp Zool 228:355–62

McGrath J, Solter D, 1984a. Maternal T^{hp} lethality in the mouse is a nuclear not cytoplasmic defect. Nature (Lond) 308:550–51

McGrath J, Solter D, 1984b. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226: 1317–19

Newport J, Kirschner M, 1982. A major transition in early *Xenopus* embryos: II. Control of the onset of transcription. Cell 30:687–96

Norberg HS, 1970. Nucleosphaerides in early pig embryos. Z Zellforsch 110: 61–71

Pope WF, Lawyer MS, First NL, 1987. The effect of exogenous estradiol on litter size in a typical swine herd. Theriogenology 28:9–14

Prather RS, 1989. Nuclear transfer in mammals and amphibians: nuclear equivalence, species specificity? In: Schatten H, Schatten G (eds.) The Molecular Biology of Fertilization. New York: Academic Press, pp. 323–40

Prather RS, Barnes FL, Sims ML, Robl JM, Eyestone WH, First NL, 1987.

Nuclear transfer in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 37:859–66
Prather RS, First NL, 1989. Nuclear transfer in mammalian embryos. Int Rev Cytol (In press)
Prather RS, Sims ML First NL, 1988. Nuclear transplantation in the porcine embryo. Theriogenology 29:290 (Abstr.)
Prather RS, Sims MM, First NL, 1989a. Nuclear transfer in pig embryos. 3rd International Conference on Pig Reproduction, April 11-14, University of Nottingham, U.K., p. 43 (Abstr.)
Prather RS, Sims MM, Maul GG, First NL, Schatten G, 1989b. Nuclear lamin antigens are developmentally regulated during porcine and bovine early embryogenesis. Biol Reprod (In press)
Robl JM, First NL, 1985. Manipulation of gametes and embryos in the pig. J Reprod Fertil 33 (Suppl.):101–14
Robl JM, Gilligan B, Critser ES, First NL, 1986. Nuclear transplantation in mouse embryos: assessment of recipient cell stage. Biol Reprod 34: 733–39
Robl JM, Lohse-Heideman JK, First NL, 1988. Strain differences in early mouse embryo development in vitro: role of the nucleus. J Exp Zool 247: 251–56
Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B, First NL, 1987. Nuclear transplantation in bovine embryos. J Anim Sci 64:642–47
Snedecor GW, Cochran WG, 1980. Statistical Methods. Ames, Iowa: Iowa State University Press, pp. 194–214
Stice SL, Robl JM, 1988. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol Reprod 39:657–64
Surani MAH, Barton SC, Norris ML, 1986. Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. Cell 45:127–36
Tsunoda Y, Shioda Y, 1988. Development of enucleated parthenogenones that received pronuclei or nuclei from fertilized mouse eggs. Gamete Res 21: 151–55
Von Beroldington CH, 1981. The developmental potential of synchronized amphibian cell nuclei. Dev Biol 81:115–26
Wall RJ, Pursel VG, Hammer RE, Brinster RL, 1985. Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol Reprod 32:645–51
White KL, Anderson GB, Berger TJ, Bon Durant RH, Pashen RL, 1987. Identification of a male specific histocompatibility protein on preimplantation porcine embryos. Gamete Res 17:107–13
Willadsen SM, 1986. Nuclear transplantation in sheep embryos. Nature (Lond) 227:298–300

NUCLEAR TRANSPLANTATION IN GOATS

Z. Yong, W. Jianchen, Q. Jufen, H. Zhiming

Laboratory of Reproductive Endocrinology in Domestic Animals, Northwestern Agricultural University 712100 Yangling, Shaanxi Province, China

It has been reported that offspring of sheep, cattle, rabbit and pig have been produced by nuclear transplantation. But a similar report has never been published for the goat. The purpose of the present study was to examine the developmental capacity of nuclear transplanted embryos produced by transfer of blastomeres from 4 to 32-cell embryos into enucleated oocytes matured in vivo.

Matured oocytes and 4-32-cell embryos were recovered surgically from superovulated Shaanbei black goats and Guanzhong Saanen dairy goats, respectively. Nuclear transplantation procedures were performed as described by Willadsen in for sheep (Nature 310:63). First the polar body, metaphase chromsomes and about half of the cytoplasm were removed from the oocyte by aspiration with a beveled glass pipette. Donor embryos were mechanically separated into individual cells by micromanipulation. Each blastomere was transferred into the zona pellucida (ZP) of the enucleated oocyte with the same beveled glass pipette. The micromanipulation was performed in phosphate buffered saline (PBS) containing 5 μg/ml cytochalasin B. The karyoplast oocyte was placed in PBS in a fusion chamber and fusion was induced with a 45 V DC pulse of 40 μsec duration. Fused nuclear transplanted embryos were inserted into another ZP and transferred after culture for 1 to 3 h in vitro in PBS with 15% fetal calf serum at 37°C.

Results of karyoplast oocyte fusion and nuclear transplanted embryo transfer

Donor embryo stage	No. attempted fusion	No. successful fusion	No. transferred embryos	No. off-spring
4-cell	7	5	5	1
8-cell	8	6	6	1
16-cell	8	7	7	1
32-cell	7	6	6	2 a)
total	30	24	24	5

a) identical twins

The present study showed that nuclear transplanted goat embryos produced by tranfer of a blastomere from 4 to 32-cell embryos into an enucleated matured oocyte can develop into normal kids in vivo; the commercial cloning of goats will be a reality in the near future.

Applicant

BIOLOGY OF REPRODUCTION 48, 958–963 (1993)

Birth of Mice after Transplantation of Early Cell-Cycle-Stage Embryonic Nuclei into Enucleated Oocytes[1]

HEE-TAE CHEONG, YOSHIYUKI TAKAHASHI, and HIROSHI KANAGAWA[2]

Department of Theriogenology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

ABSTRACT

The present study was conducted to investigate the influence of cell cycle stage of the donor nucleus on chromatin structure and development of mouse embryonic nuclei transplanted into enucleated oocytes. Donor cell-cycle stage was controlled in order to examine, in addition, the developmental potential of nuclei from 2-, 4-, and 8-cell-stage embryos. The cell cycle stage of donor nuclei was classified as early, middle, or late. After nuclear transfer, electrofusion, and activation, early-stage transplants formed a single pronucleus-like structure, but middle-stage transplants formed very irregular types of structures and late-stage transplants extruded a polar body. A high proportion of development to the blastocyst stage (77.8%) and an increased cell number (62.1 cells) were obtained from the early 2-cell-stage transplants as opposed to the middle- (0%) and late-stage (20.8%, 37.0 cells) transplants ($p < 0.001$). With transplantation of early-stage nuclei, high proportions of development to the blastocyst stage and of offspring were obtained from nuclear transplant embryos with a nucleus from a 2-, 4-, or 8-cell-stage embryo. The results confirm that the donor cell-cycle stage critically affects the chromatin structure and development of nuclear transplant embryos. The results also demonstrate that the nuclei from 2-, 4-, and 8-cell-stage mouse embryos in the early stage of each cell cycle can be reprogrammed when transplanted into enucleated mature oocytes.

INTRODUCTION

A nucleus transplanted into an enucleated mature oocyte undergoes various morphological modifications. These changes are characterized by a premature chromosome condensation (PCC) followed by pronucleus-like formation and swelling of the nucleus upon activation of the oocyte [1, 2]. PCC and nuclear swelling have been considered morphological signs of the reprogramming of transferred nuclei (reviewed in [3, 4]).

In somatic cell hybrids, the morphology of the PCC depends on the cell cycle stage of the donor nucleus. PCC in the G1 and G2 phases results in elongated chromosomes with single- and double-stranded chromatids, respectively. However, PCC in the S phase leads to heterogeneous chromatin elements with small dispersed fragments [5, 6]. Furthermore, PCC in different cell-cycle stages influences spindle structure, chromosome constitution, and in vitro development of nuclear transplant rabbit embryos [7, 8]. It has been reported that spindle and chromosome constitution are normal and that development to the blastocyst stage is improved when blastomere nuclei in G1 phase are transplanted to mature, enucleated oocytes. However, S-phase nuclear-transplant embryos develop poorly compared to G1 phase transplants. Thus, it has been suggested that synchronization of the donor nucleus in the G1 phase is an important factor for successful development of nuclear transplant embryos [7, 8].

The influence of donor cell-cycle stage on developmental capacity of mouse blastomere nuclei transplanted into enucleated oocytes has also been reported [9]. In the mouse, nuclear transplant embryos extrude an extra polar body regardless of the cell cycle stage of the donor nucleus, but full-term development is achieved only by nuclear transplant embryos that received late 2-cell-stage (presumably G2 phase) donor nuclei [9].

The present study was conducted to examine the influence of donor cell-cycle stage on the chromatin structure of the donor nucleus and the development of nuclear transplant mouse embryos. Donor cell-cycle stage was controlled in order to investigate, in addition, the developmental potential of nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

F1 hybrid (C57BL/6J × CBA) and ICR strain female mice were superovulated with injections of 5 IU of eCG (Serotropin; Teikoku Zoki, Tokyo, Japan), followed 48 h later by 5 IU of hCG (Gonatropin; Teikoku Zoki). Oocytes at metaphase (M phase) II were collected from the ampullae of the oviducts of F1 females 14–15 h after hCG injection with 37°C M2 medium [10]. Cumulus cells were removed by treatment with 300 IU/ml hyaluronidase (Sigma, St. Louis, MO).

To collect donor embryos, the oviducts of ICR females mated with F1 strain males were flushed using 37°C M2 medium. Late 1-cell-stage embryos were collected 29–30 h after hCG injection. Two-cell-stage embryos were collected 44–45 and 48–49 h after hCG injection, respectively. Late 4-cell-stage embryos were collected 59–60 h after hCG injection. The zona pellucidae of late 4-cell-stage embryos were removed after a short pretreatment with 0.5% pronase (A

Accepted December 11, 1992.
Received October 13, 1992.
[1]This study was supported by Grants-in-Aid for Scientific Research (No. 02556039) from the Ministry of Education, Science and Culture, Japan.
[2]Correspondence. FAX: (011) 717-7569.

tinase; Kaken, Tokyo, Japan) in 37°C BSA-free M2 medium. Blastomeres of 4-cell embryos were isolated by pipetting zona-free embryos in Ca^{2+}-free M16 medium [11].

Enucleation of Oocytes

All manipulations were performed via an inverted microscope (Diaphot; Nikon, Tokyo, Japan) with Nomarski optics and Narishige micromanipulators at room temperature (25–28°C) for 1–1.5 h. Oocytes were enucleated by removal of the M phase chromosomes arranged in the spindle of the second meiotic division. This system had provided a high enucleation rate (89%) in our previous study (Cheong et al., unpublished results). Oocytes were placed in a drop of M2 medium in a petri dish covered with paraffin oil. The zona pellucidae over the spindle area were slit with a fine glass needle along 10–20% of their circumference. After the zona were slit, the oocytes were placed in a drop of M2 medium containing 5 μg/ml cytochalasin B (CB; Sigma) and 0.1 μg/ml colcemid (Gibco, Grand Island, NY). The oocytes were secured by a holding pipette opposite the slit in the zona. An enucleation pipette (20–25 μm in diameter) with a beveled sharpened tip was inserted into the perivitelline space through the slit, and the spindle area of M phase chromosomes was aspirated into the enucleation pipette with a small amount of cytoplasm.

Nuclear Transplantation

Nuclear transplantation was carried out as described previously [12, 13]. A karyoplast from 2- and 4-cell-stage embryos or a blastomere from 8-cell donor embryos was introduced into the perivitelline space of the enucleated oocytes. The manipulated eggs were then cultured in M16 medium containing 100 μM EDTA in an atmosphere of 5% CO_2 in air at 37°C for 10–30 min before fusion treatment.

Electrofusion and Activation

Membrane fusion was performed by electrofusion as described previously [14], 18–20 h after hCG injection of the oocyte donors. Manipulated eggs were placed in a 0.5-mm round wire, stainless steel electrode chamber filled with 37°C 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.05 mg/ml of BSA. The eggs were aligned by exposure to an alternating current (a.c.) pulse of 0.6 MHz, 6 V, for 6 sec; then two direct current (d.c.) pulses of 1.25 kV/cm for 70 μsec (each pulse 1 sec apart) were applied to the chamber via an Electro Cell Fusion instrument (LF 100; Life Tec, Tokyo, Japan). After fusion treatment, eggs were placed in culture (see below) and checked every 10 min. Manipulated embryos were fused usually within 15 min and were undergoing PCC within 30 min. At around the time of onset of PCC (25–30 min after fusion treatment), fused embryos were treated again with 5–6 d.c. pulses of 1.5 kV/cm for 70 μsec (each pulse 1 sec apart) to obtain sufficient activation of oocytes. In a separate experiment, with two d.c. pulses of 1.25 kV/cm for 70 μsec, 90% (27/30) of nuclear transplant mouse embryos were fused and 11% (3/27) of fused embryos were activated, showing a pronucleus-like formation. However, when an additional five to six d.c. pulses of 1.5 kV/cm for 70 μsec were given after fusion treatment of two d.c. pulses of 1.25 kV/cm for 70 μsec, 97% (30/31) of fused embryos were activated, showing a pronucleus-like formation (unpublished data). The average time from oocyte collection until electrofusion with donor cells was 4–5 h.

In Vitro Culture, Monitoring, and Embryo Transfer

After fusion and activation treatments, the nuclear transplant embryos were cultured in drops of M16 medium containing 100 μM EDTA and covered with paraffin oil, in an atmosphere of 5% CO_2 in air at 37°C. Embryos were monitored every hour for 4 h to assess activation, and their development in vitro was assessed every 8 h. Activated embryos were those showing pronucleus-like formation 3–4 h after fusion treatment [9]. Nuclear transplant embryos that had developed to the blastocyst stage by 96 h after culture were transferred to the uterine horn of Day 3 (2.5 days post-coitum) pseudopregnant recipients (ICR strain). Control blastocysts derived from ICR females mated with ICR males were transferred into the same recipients. The recipients were retained until parturition.

Preparations

Whole-mount preparations were made through use of the method of Bedford [15]. Embryos were mounted on a slide, fixed with 10% neutral formalin for 2 h, and stained with 0.25% aceto-lacmoid. Air-dried preparations were made according to Tarkowski [16]. Blastocysts were incubated in M16 medium containing 0.4 μg/ml colchicine for 3—4 h to arrest cleavage division at M phase. They were then treated with a hypotonic solution of 1% sodium citrate for 15 min, fixed with a mixture of methanol and acetic acid (3:1), and stained with 4% Giemsa.

Experiment 1

The first experiment investigated the effect of donor cell-cycle stage on the type of pronucleus-like formation and in vitro development of nuclear transplant embryos. Late 1-cell-stage embryos were collected, and most of these were cultured in M16 medium containing 0.1 μg/ml colcemid (Sigma) at 37°C for 1–2 h. Some embryos already in mitosis were stored at 4°C in M2 medium for the same period. Colcemid or cold treatment of embryos for a short time had no adverse effect on development to the blastocyst stage in vitro. Embryos were removed from colcemid or cold and were placed in culture (see above). Cleavage to the 2-cell stage was assessed every 15 min, and newly cleaved embryos at 0.5–1.5 h after release from colcemid or cold were assigned to an experimental treatment. Embryos were im-

TABLE 1. Influence of donor cell-cycle stage on fusion and oocyte activation of manipulated eggs with nuclei from 2-cell-stage embryos.

Donor cell-cycle stage	Time[a] of activation	No. (%) of oocytes fused/ manipulated[b]	No. (%)[c] of oocytes activated	No. (%) of activated embryos with [d]			
				1PN	1PN + 1PB	1PN + 2PB ≤	Others
Early	1.5 hpc	37/80(46.3)[e]	36(97.3)	35(97.2)[e]	0[e]	0[e]	1(2.8)[e]
Middle	5-6 hpc	70/82(85.4)[f]	68(97.1)	6(8.8)[f]	17(24.3)[f]	16(22.9)[f]	31(45.6)[f]
Late	47-48 hph	78/86(90.7)[f]	77(98.7)	4(5.2)[f]	52(67.5)[g]	15(19.5)[f]	6(7.8)[e]

[a]hpc: hours post-cleavage; hph: hours post-hCG injection.
[b]Total of five replicates.
[c]Based on the number of fused embryos.
[d]PN: pronucleus; PB: polar body.
[e-g]Values with different superscripts in the same column are significantly different ($p < 0.01$).

mediately used (early stage) or cultured in M16 medium containing 100 μM EDTA at 37°C for 3–4 h before nuclear transfer (middle stage). Nuclear transfer, fusion, and activation were completed within 1.5 and 5–6 h after cleavage for early- and middle-stage donors, respectively. These times are likely to be associated with the G1 and S phases of the second cell cycle, respectively [17, 18]. Two-cell-stage embryos collected 44–45 h after hCG injection were stored at 4°C before nuclear transfer and fusion (47–48 h after hCG injection; late stage). This timing is likely to be associated with the G2 phase of the second cell cycle [18]. Nuclear transplant embryos were monitored every hour for 4 h for assessment of activation and type of pronucleus-like formation. Activated embryos were cultured in vitro for 4 days for assessment of their developmental potential in vitro. Five replicates were performed.

Experiment 2

The chromatin structure of the donor nucleus after fusion treatment was investigated. Nuclei from 2-cell-stage embryos in different stages of the cell cycle were transferred and fused to enucleated oocytes. About 1.5 h after fusion treatment, embryos were prepared via the whole-mount technique and examined under a microscope equipped with Nomarski optics.

Experiment 3

The potential of donor nuclei in the early cell-cycle stage to direct full-term development of nuclear transplant embryos was determined. The early stage of the second cell cycle was determined as described for experiment 1. Early stages of the third and fourth cell cycles were determined by time after cleavage. Two-cell-stage embryos collected 48–49 h after hCG injection and blastomeres of late 4-cell-stage embryos received low-temperature or colcemid treatment, and cleavage was assessed after release from these treatments as described for experiment 1. Cleaving blastomeres of late 4-cell-stage embryos were isolated by pipetting in Ca^{2+}-free M16 medium. Nuclear transfer, fusion, and activation were completed within 1.5 h after cleavage for early 4- and 8-cell donor nuclei to adjust cell stage to the G1 phases of the third [17, 19] and fourth cell cycles [19], respectively. Nuclear transplant embryos were cultured in vitro for 4 days and blastocysts were transferred to recipient females. Six replicates were performed.

Statistical Analysis

Data were analyzed by chi-square test or Student's *t*-test.

RESULTS

Experiment 1

Fusion rate was affected by the donor cell-cycle stage. A significantly lower ($p < 0.01$) fusion rate was obtained in the early stage (46.3%) than in the middle (85.4%) and late stages (90.7%). Most of the fused eggs were activated regardless of donor cell-cycle stage. However, types of activation varied and were significantly ($p < 0.01$) affected by the stage of the donor cell cycle (Table 1). Most of the nuclear transplant embryos with nuclei in the early cell-cycle stage (97.2%) showed a single pronucleus-like (1PN) structure. However, transplantation of middle-stage donor nuclei yielded a variety of complex and irregular structures. Transplantation of late-stage donor nuclei resulted primar-

TABLE 2. In vitro development of nuclear transplant embryos with nuclei from 2-cell-stage embryos in different cell-cycle stages.

Donor cell-cycle stage	No. of embryos cultured	No. (%) of embryos developed to					Cell number in blastocysts (mean ± SEM)
		2-cell	4-cell	8-cell	Morulae	Blastocyst	
Early	36	35(97.2)[a]	33(91.7)[d]	31(86.1)[d]	30(83.3)[d]	28(77.8)[d]	62.1 ± 2.2[d]
Middle	68	26(38.2)[b]	5(7.4)[e]	0[e]	0[e]	0[e]	—
Late	77	60(77.9)[c]	34(44.2)[f]	25(32.5)[f]	19(24.7)[f]	16(20.8)[f]	37.0 ± 1.6[f]

[a-c]Values with different superscripts are significantly different ($p < 0.05$).
[d-f]Values with different superscripts in the same column are significantly different ($p < 0.001$).

TABLE 3. Chromatin structure of nuclear transplant embryos in whole-mounts 1.5 h after fusion treatment.

Donor cell-cycle stage	No. of embryos analyzed	No. (%) of embryos with different no. of chromatin clumps			
		1	2	3	>3 clumps
Early	20	20(100.0)[a]	0[a]	0[a]	0[a]
Middle	27	2(7.4)[b]	6(22.2)[a]	9(33.3)[b]	10(37.0)[b]
Late	28	1(3.6)[b]	18(64.3)[b]	8(28.6)[b]	1(3.6)[a]

[a,b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

ily (67.5%) in the formation of 1PN structure as well as a polar body (1PN + 1PB).

The developmental capacity of nuclear transplant embryos to the blastocyst stage was significantly ($p < 0.01$) affected by the donor cell-cycle stage (Table 2). A very high proportion (97.2%) of embryos with donor nuclei in the early stage of the cell cycle cleaved, and 77.8% of cultured embryos with these nuclei developed to the blastocyst stage. With donor nuclei in the middle and late stages of the cell cycle, however, the proportions of development to the 2-cell stage (38.2% and 77.9%, respectively, $p < 0.05$) and to the blastocyst stage (0% and 20.8%, respectively, $p < 0.001$) were significantly reduced as compared to those for the early-stage nuclei.

Mean cell number in blastocysts after 96 h of culture was significantly lower ($p < 0.001$) for nuclei in the late cell-cycle stage (37.0 cells) than for nuclei in the early cell-cycle stage (62.1 cells). All embryos with early cell-cycle-stage nuclei that were analyzed (21/21) had a diploid chromosome constitution. However, 23% (3/13) of embryos with nuclei in the late cell-cycle stage had a tetraploid chromosome constitution.

Experiment 2

Chromatin structure of the donor nucleus was determined in whole mounts 1.5 h after fusion treatment (Table 3). All embryos with nuclei in the early stage of the cell cycle had one clump of chromatin in their cytoplasm (Fig. 1A). However, most of the embryos with nuclei in the middle stage of the cell cycle (70.3%) had three or more clumps of chromatin irregularly dispersed in their cytoplasm (Fig. 1B). Most of the embryos with nuclei in the late stage of the cell cycle (64.3%) showed normal mitosis and had two clumps of chromatin (Fig. 1C).

Experiment 3

The developmental potential of 2-, 4-, and 8-cell donor nuclei in the early stage of the cell cycle was examined (Table 4). The rates of fusion, activation, and cleavage were not affected by donor cell stage. Development to the blastocyst stage was similarly high with 2- and 4-cell-stage donor nuclei (78.2% and 71.4%, respectively). However, the developmental potential of 8-cell nuclear donors was sig-

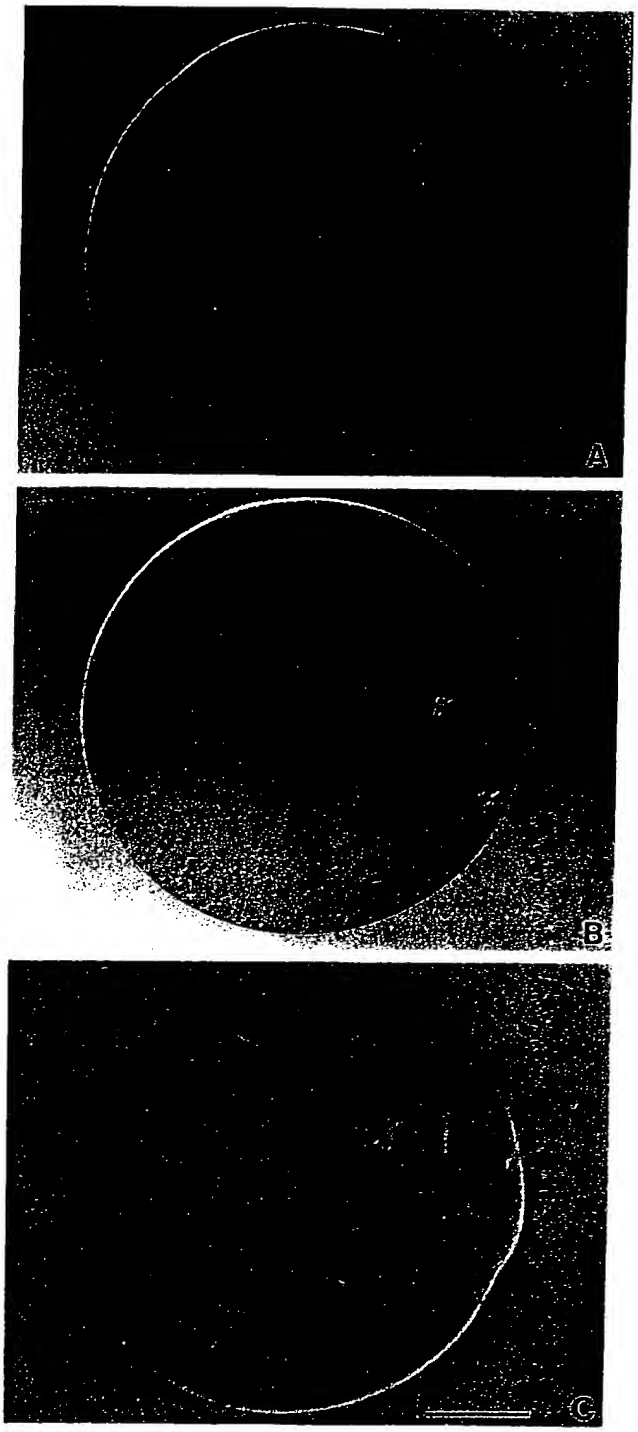


FIG. 1. Chromatin structures of nuclear transplant embryos 1.5 h after fusion treatment. A) Early-stage transplants: one clump of chromatin is seen in the cytoplasm. B) Middle-stage transplants: several clumps of chromatin are dispersed in the cytoplasm; some chromatin clumps are out of focus. C) Late-stage transplants: a normal telophase plate is shown in the cytoplasm. The bar represents 40 μm.

TABLE 4. Development of nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos in the early stage of the cell cycle.

Stage of donor nuclei	No. (%) of oocytes fused/ manipulated[a]	No. (%) of oocytes activated[b]	No. (%) of embryos developed to		No. (%) pregnant/ no. of recipient	No. (%) of young/ no. of embryos transferred
			2-cell	Blastocyst		
2-cell	48/102(47.1)	46(95.8)	45(97.8)	36(78.2)[c]	5/8(62.5)	10/34(29.4)
4-cell	43/ 97(44.3)	42(97.7)	42(100.0)	30(71.4)[c]	4/6(66.7)	6/27(22.2)
8-cell	41/ 94(43.6)	39(95.1)	36(92.3)	18(46.2)[d]	3/6(50.0)	3/17(17.6)

[a]Total of six replicates.
[b]Embryos were activated within 1.5 h post-cleavage for each stage of the donor nuclei; percentages were based on the number of fused embryos.
[cd]Values with different superscripts are significantly different ($p < 0.01$).

nificantly reduced (46.2%, $p < 0.01$). After transfer of blastocysts derived from nuclear transplant embryos to pseudopregnant recipients, 10 (29.4%), 6 (22.2%), and 3 (17.6%) live young were obtained from embryos with 2-, 4-, and 8-cell-stage donor nuclei, respectively. Of the 86 transferred blastocysts derived from ICR mice, 39 (45.3%) developed to term.

DISCUSSION

The results of the present study confirm that the cell cycle stage of the donor nucleus affects the chromatin structure of the donor nucleus and the developmental ability of nuclear transplant embryos. The nucleus in the early stage of the cell cycle demonstrated great developmental potential to the blastocyst stage. Furthermore, successful development to the blastocyst stage and to term was achieved from transplantation of nuclei from 2-, 4-, and 8-cell-stage embryos in the early stage of each cell cycle, strongly confirming complete reprogramming. To our knowledge this is the first time this has been achieved.

As no direct observations of cell cycle stage were made in the donor nuclei in this study, interpretation of these results depends upon earlier experiments in other laboratories. However, it seems very likely that the stages described as early, middle, and late in our experiments corresponded to G1, S, and G2, respectively. Cell cycle stage of mouse embryos has been established for the first [20, 21], second [17, 18], third [17, 19], and fourth [19] cell cycles.

In the present study, in which the time after cleavage was controlled, high proportions of development to the blastocyst stage as well as a large increase in cell number of blastocysts were obtained in nuclear transplant embryos with nuclei in the early stage of the cell cycle, although the fusion rate was low. The greater developmental potential of nuclear transplant embryos with early-stage donor nuclei may be explained by the pronucleus-like formation type, whole-mount, and chromosome preparations. The early-stage transplantations showed 1PN structure after activation and had a diploid chromosome constitution. Most of the late-stage transplantations showed 1PN + 1PB structure after activation, and some embryos developed to the blastocyst stage with diploid chromosomes. However, our previous findings (Cheong et al., unpublished results) confirmed that the chromosome number of late-stage transplants with 1PN + 1PB varied at the 1-cell stage, and only a small proportion (37.5%) of these nuclear transplant embryos had a diploid chromosome constitution. Chromosome abnormalities in the middle-stage transplants are also consistent with the data on pronucleus-like formation and the whole-mount preparation. A large proportion of chromosome abnormalities were also observed in late S phase transplants in the rabbit [8].

The results of the current study are in contrast with those from a previous mouse nuclear-transplant study [9]. In that investigation most of the nuclear transplant embryos emitted a polar body after activation regardless of the donor cell-cycle stage, and no development to the blastocyst stage or to term was obtained from the early-stage transplants. This discrepancy is presumably due to differences either in the stage of recipient oocytes used or in activation treatment and time. In the present study, M phase II oocytes were used for recipient cells, and electric field-induced fusion and activation were completed within 1.5 h after cleavage for early-stage transplantation. In the study by Kono et al. [9], however, telophase I oocytes were employed for recipient cells, and activation treatment was performed with ethanol at 1.5 h after virus-induced fusion. On the other hand, the fact that pronucleus-like formation type varies with different species cannot be disregarded. The extrusion of a polar body was not observed after activation in rabbits [2, 7, 8, 22], sheep [23, 24], or cattle [25, 26].

In the mouse, 2-cell-stage nuclei transferred to enucleated zygotes [27] and oocytes [9] can develop to term, but the developmental potential of nuclei from 4- or 8-cell-stage embryos is seriously limited. No offspring have been obtained after transfer of these nuclei to enucleated zygotes [13, 27, 28] or oocytes [29, 30]. It has been suggested that the ability of nuclear transplant embryos to develop to the blastocyst stage is affected by the transcriptional activity of the embryonic genome [31], which occurs at the middle 2-cell stage in the mouse [32, 33]. In the present study, however, not only high proportions of development to the blastocyst stage but also normal live young were successfully obtained from the nuclear transplant embryos with nuclei from 2-, 4-, and 8-cell-stage embryos. These results suggest that initiation of transcription has little effect on the devel-

opmental potential of donor nuclei transplanted into enucleated mature oocytes, although reduced development in vitro was observed with the use of an 8-cell nucleus donor. This suggestion is supported by findings in rabbits [2], sheep [23, 24], and cattle [25]. In these species, pre- and post-transcriptional nuclei directed development of nuclear transplant embryos to the morula or blastocyst stage in similar proportions. Reduction in the developmental potential of 8-cell donor nuclei may have been due either to intensive pipetting to separate blastomeres immediately after cleavage or to the advanced transcriptional age of the donor nuclei rather than to transcriptional activation itself.

ACKNOWLEDGMENTS

We wish to thank Dr. A.A. Rayos for helpful evaluation of the manuscript and Dr. J.I. Park for preparation of the disk version.

REFERENCES

1. Czolowska R, Modlinski JA, Tarkowski AK. Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. J Cell Sci 1984; 69:19–34.
2. Collas P, Robl JM. Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. Biol Reprod 1991; 45:455–465.
3. Prather RS, First NL. Cloning embryos by nuclear transfer. J Reprod Fertil Suppl 1990; 41:125–134.
4. Prather RS, First NL. Nuclear transfer in mammalian embryos. Int Rev Cytol 1990; 120:169–190.
5. Johnson RT, Rao PN. Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. Nature 1970; 226:717–722.
6. Rao PN, Johnson RT. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 1970; 225:159–164.
7. Collas P, Balise JJ, Robl JM. Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. Biol Reprod 1992; 46:492–500.
8. Collas P, Pinto-Correia C, Ponce De Leon FA, Robl JM. Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. Biol Reprod 1992; 46:501–511.
9. Kono T, Kwon OY, Watanabe T, Nakahara T. Development of mouse enucleated oocytes receiving a nucleus from different stages of the second cell cycle. J Reprod Fertil 1992; 94:481–487.
10. Fulton BP, Whittingham DG. Activation of mammalian oocytes by intracellular injection of calcium. Nature 1978; 273:149–151.
11. Whittingham DG. Culture of mouse ova. J Reprod Fertil Suppl 1971; 14:7–21.
12. McGrath J, Solter D. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 1983; 220:1300–1302.
13. McGrath J, Solter D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 1984; 226:1317–1319.
14. Cheong HT, Taniguchi T, Hishinuma M, Takahashi Y, Kanagawa H. Effects of various electric fields on the fusion and in vitro development of mouse two-cell embryos. Theriogenology 1991; 36:875–885.
15. Bedford JM. Techniques and criteria used in the study of fertilization. In: Daniel JC (ed.), Methods in Mammalian Embryology. San Francisco: WH Freeman; 1971: 37–63.
16. Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 1966; 5:394–400.
17. Sawicki W, Abramczuk J, Blaton O. DNA synthesis in the second and third cell cycles of mouse preimplantation development. Exp Cell Res 1978; 112:199–205.
18. Bolton VN, Oades PJ, Johnson MH. The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol 1984; 79:139–163.
19. Smith RKW, Johnson MH. Analysis of the third and fourth cell cycles of mouse early development. J Reprod Fertil 1986; 76:393–399.
20. Abramczuk J. Sawicki W. Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs. A cytogenetic study. Exp Cell Res 1975; 92:361–372.
21. Howlett SK, Bolton VN. Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. J Embryol Exp Morphol 1985; 87:175–206.
22. Stice SL, Robl JM. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol Reprod 1988; 39:657–664.
23. Willadsen SM. Nuclear transplantation in sheep embryos. Nature 1986; 320:63–65.
24. Smith LC, Wilmut I. Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. Biol Reprod 1989; 40:1027–1035.
25. Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 1987; 37:859–866.
26. Bondioli KR, Westhusin ME, Looney CR. Production of identical bovine offspring by nuclear transfer. Theriogenology 1990; 33:165–174.
27. Tsunoda Y, Yasui T, Shioda Y, Nakamura K, Uchida T, Sugie T. Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. J Exp Zool 1987; 240:119–125.
28. Howlett SK, Barton SC, Surani MAH. Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. Development 1987; 101:915—923.
29. Tsunoda Y, Tokunaga T, Imai H, Uchida T. Nuclear transplantation of male primordial germ cells in the mouse. Development 1989; 107:407–411.
30. Kono T, Kwon OY, Nakahara T. Development of enucleated mouse oocytes reconstituted with embryonic nuclei. J Reprod Fertil 1991; 93:165–172.
31. Surani MAH, Barton SC, Norris ML. Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. Biol Reprod 1987; 36:1–16.
32. Piko L, Clegg KB. Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. Dev Biol 1982; 89:362–378.
33. Bensaude O, Babinet C, Morange M, Jacob F. Heat shock proteins, first major products of zygotic gene activity in mouse embryo. Nature 1983; 305:331–333.

Applicant

BIOLOGY OF REPRODUCTION 47, 636–643 (1992)

Nuclear Totipotency of Cultured Rabbit Morulae to Support Full-Term Development Following Nuclear Transfer[1]

XIANGZHONG YANG,[2] SHI-E JIANG, ANDRÁS KOVÁCS, and ROBERT H. FOOTE

Department of Animal Science, Cornell University, Ithaca, New York 14853–4801

ABSTRACT

The rabbit was used as a model for nuclear transfer. A critical step in nuclear transfer is oocyte activation, which was evaluated in this research. Optimal field strength of an electric stimulus for activation was examined. A significantly higher activation rate in all criteria tested was achieved when oocytes were activated electrically with a field strength of 2.4 kV/cm versus 1.2 or 1.8 kV/cm. Also, electrical stimulation with combined alternating current (AC) and direct current (DC) was superior to DC stimulation alone for activation. In another study involving 586 oocytes, exposure of oocytes to cytochalasin B for 1 h followed by activation with electrical stimulation significantly improved development of the oocytes to blastocyst stage compared to oocytes without cytochalasin B pre-exposure (38% vs. 26%, $p < 0.05$). Cytochalasin B exposure alone (control), however, had no effect on activation. Exposing oocytes to activation medium without electrical stimulation also activated some oocytes.

In the nuclear transfer experiment, blastomeres from 8-cell embryos cultured for 20–24 h to the 32–64-cell stage were used as nuclear donor cells. Of 491 oocytes used, 459 (93%) survived the enucleation and fusion procedure, 370 (81%) fused, and 284 (77%) developed into 2–4-cell embryos. A total of 243 of these 2–4-cell embryos were transferred to 15 pseudopregnant recipients and produced 8 young (3%). Although the efficiency is low, this study demonstrated that rabbit morulae cultured for 20–24 h to the 32–64-cell stage as nuclear donors for transfer remain totipotent.

INTRODUCTION

Nuclear transfer to clone mammalian preimplantational embryos is important scientifically for evaluating nuclear totipotency and nuclear-cytoplasmic interactions. It is potentially one of the most powerful tools for producing lines of animals having the desired sex and other characteristics. Multicellular embryos retain totipotency of each blastomere at the 4–16-cell stage as shown by nuclear transfer in sheep [1], cattle [2, 3], rabbits ([4, 5]; Yang and Foote, unpublished data), and pigs [6]. Likewise, morulae and blastocysts in cattle [3, 7] and sheep [8] maintain some nuclear totipotency. However, the proportion of the nuclear transferred embryos developing into offspring is only 1–4% in these species [5, 9].

Because the genomic shift from maternal to zygotic control of development in rabbits is similar to that in cattle, the rabbit may be a good model for research on nuclear transfer in cattle. Additionally, embryos at precise stages can be obtained easily because rabbits are induced ovulators. In addition, rabbit embryos can be cultured in vitro in relatively simple medium from zygotes to blastocysts with no culture block. In this study, we examined nuclear totipotency by nuclear transfer of rabbit morulae (32–64 cells) produced from 8-cell embryos by culturing for 20–24 h.

Accepted June 15, 1992

Received June 7, 1991.

[1]This research was partially supported by The Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation; and Eastern A. I. Cooperative, Inc. Preliminary results of this research were presented at the 1991 International Embryo Transfer Society Meeting, January, 1991, Bournemouth, England.

[2]Correspondence: Dr. X. Yang, 227 Morrison Hall, Animal Science Department, Cornell University, Ithaca, NY 14853–4801. FAX: (607) 255–9829.

MATERIALS AND METHODS

Oocytes and Embryo Donors

All animals used in these studies were sexually mature Dutch-belted rabbits. Young does (5 mo old) were primed with 1.2 μg of GnRH analog (Buserelin, Hoechst-Roussel Agri-Vet Co., Somerville, NJ) at least 1 mo before use as donor animals. This was to develop the reproductive system by exposure to progesterone before use. Donor does were superovulated with FSH and LH (LH, Burns-Biotech, Inc., Omaha, NE), as reported previously [10, 11]. Freshly ovulated oocytes were collected surgically approximately 14 h after LH administration by flushing oviducts through the uterotubal junction and collecting the fluid through a cannula clamped in the infundibulum. These oocytes served as recipients of transferred blastomeres. Donor embryos at the 8-cell stage were collected similarly from the oviducts 38–40 h after LH injection and insemination. Three to five embryos were selected and cultured in Medium 199 with Earle's salts (Gibco, Grand Island, NY) containing 15% fetal calf serum (M199-FCS, Gibco) for 20–24 h until used as nuclear donors. At this time they consisted of 32–64 blastomeres.

Nuclear Transfer Manipulation

Nuclear transfer manipulation involves (1) isolation of embryo blastomeres (nuclear donors), (2) preparation and enucleation of matured oocytes (nuclear recipients), (3) insertion of a blastomere into the perivitelline space of an enucleated oocyte, and (4) membrane fusion between the blastomere and the oocyte and activation induced by electric pulses. Freshly ovulated oocytes were freed of cumulus cells by pipetting or vortexing in calcium-free Dulbecco's

PBS (DPBS) containing 0.4% BSA and 0.2% hyaluronidase. Cumulus-free oocytes were placed into preincubated (incubated in a 5% CO_2 incubator at 39°C approximately 1–2 h before micromanipulation) M199-FCS droplets containing 7.5 μg/ml cytochalasin B for 10–15 min. During this time, cultured morulae (32–64 cells) were freed of their zonae pellucidae and mucin coats as reported by Yang et al. [12]. The zona-free embryos (3–5 embryos in each group) were placed into the micromanipulation droplet containing the oocytes. Blastomeres were separated by pipetting with a holding pipette and pooled. Oocytes were enucleated by aspirating the polar body and the adjacent cytoplasm, presumably containing the metaphase plate [4, 5]. For visualization of the polar body during enucleation, the oocyte was fixed onto the holding pipette with the polar body away from the holding pipette and approximately 20° from the axis of the pipette. The zona pellucida was penetrated with a beveled enucleation pipette along the axis of the holding pipette with the bevel facing the polar body. To speed up micromanipulation, metaphase chromosomes were not examined during enucleation. However, in another series of studies, enucleated oocytes were evaluated for the presence of chromatin to determine efficiency of removal of the oocyte nucleus. All enucleated oocytes (Fig. 1D) were used for nuclear transfer. Only healthy-looking blastomeres were selected for transfer. A single blastomere was inserted into the perivitelline space of an enucleated oocyte through the original puncture in the zona pellucida.

Oocyte Activation: Effect of Field Strength

The source of electric current supply for oocyte activation was a BTX Electro Cell Manipulator 200 electrofusion apparatus monitored with a BTX Optimizer Graphic Pulse Analyzer (BTX, San Diego, CA). The activation chamber was a BTX cell fusion chamber 450 (BTX) built with two electrodes 0.5 mm apart. The first experiment was conducted to examine the optimal field strength of the electric pulse (direct current, DC). Oocytes to be activated were washed and equilibrated in 0.3 M mannitol in water for 10 min. The oocytes (10–15 at one time) were then placed into the activation chamber overlaid with the activation medium and were given a single DC pulse of 0, 1.2, 1.8, or 2.4 kV/cm field strength.

Oocyte Activation: Effect of Extracellular Ions and Alternating Current (AC) Stimulation

The effects of supplementing the activation medium with calcium and magnesium ions and of introducing an additional AC pulse (0.1 kV/cm for 5 sec) prior to the DC pulse (2.4 kV/cm for 60 μsec) on the rate of activation were also tested. A 2 × 2 factorial experimental design was employed to compare activation medium of mannitol (0.3 M) alone (M−) versus mannitol with 0.05 mM $CaCl_2$ and 0.10 mM $MgSO_4$ (M+), and electric stimulus with DC alone (2.4 kV/cm) versus DC with an additional AC stimulus (0.1 kV/cm) immediately before the DC pulse.

Oocyte Activation: Effect of Cytochalasin B

Because cytochalasin B is often used in the current nuclear transfer scheme, its effect on oocyte activation was examined. In this experiment, oocytes in replicates 7–10 were assigned to the following treatments: (1) oocytes were placed into a preincubated micromanipulation droplet (described previously) containing 7.5 μg/ml of cytochalasin B as during nuclear transfer manipulation for approximately 1 h and then were exposed to fusion medium followed by electric pulses; (2) oocytes were held in micromanipulation droplets containing no cytochalasin B for 1 h and then were exposed to fusion medium for 10 min, followed by activation as in the first treatment; (3) oocytes were treated in cytochalasin B for 1 h as in treatment 1, but without receiving electric stimulation and mannitol exposure (cytochalasin B control); (4) oocytes were treated as in treatment 2, but without activation treatment (mannitol control); and (5) oocytes were held in DPBS throughout the whole procedure (negative control). The discrepancy in the total numbers of observations was due to varied numbers of replications.

Membrane Fusion

Membrane fusion between the blastomere and the enucleated oocyte was accomplished by electrofusion similar to that of the activation treatment. The following modifications to our previous procedure for fusion were made on the basis of the activation results of the present study and those reported previously [5, 8, 12, 13]: (1) 0.3 M M+ was used as fusion medium; (2) an AC alignment pulse was applied immediately before a DC pulse to induce alignment without mechanical means. For the fusion procedure, blastomere/oocyte complexes (BOC) were initially held in DPBS. An equal volume of fusion medium was then added. This was allowed to equilibrate for 5 min, and the BOC were then placed into fusion medium. Groups of 3–5 BOC were placed between the two electrodes (0.5 mm apart) of a BTX fusion chamber (BTX) overlaid with fusion medium. The BOC were aligned by 0.1–0.15 kV/cm, 1.0 MHz AC current for 5–15 sec until alignment was observed; a DC pulse of 2.4 kV/cm for 60 μsec was applied to induce fusion. To avoid loss of blastomeres through the zona, the BOC were transferred to a small drop of fusion medium and then gradually exposed to DPBS as described above.

Embryo Culture

Embryos (micromanipulated or control zygotes) were cultured in M199-FCS droplets covered with Dow Corning Medical Fluid 360 (Dow Corning, Midland, MI) in 5% CO_2:95% humidified air at 39°C for 20–22 h. Cleavage (2–4-cell stage) was evaluated, and good-quality embryos were

FIG. 1. Enucleation procedure used to prepare the recipient oocyte for blastomere (nuclear) transfer (see text for details): A) Matured oocyte on a holding pipette with a polar body in the perivitelline space. ×350. B) Removal of the polar body and adjacent cytoplasm containing nuclear material by aspiration. ×350. C) A group of matured oocytes with the first polar body. ×175. D) Same group of oocytes after enucleation. ×175.

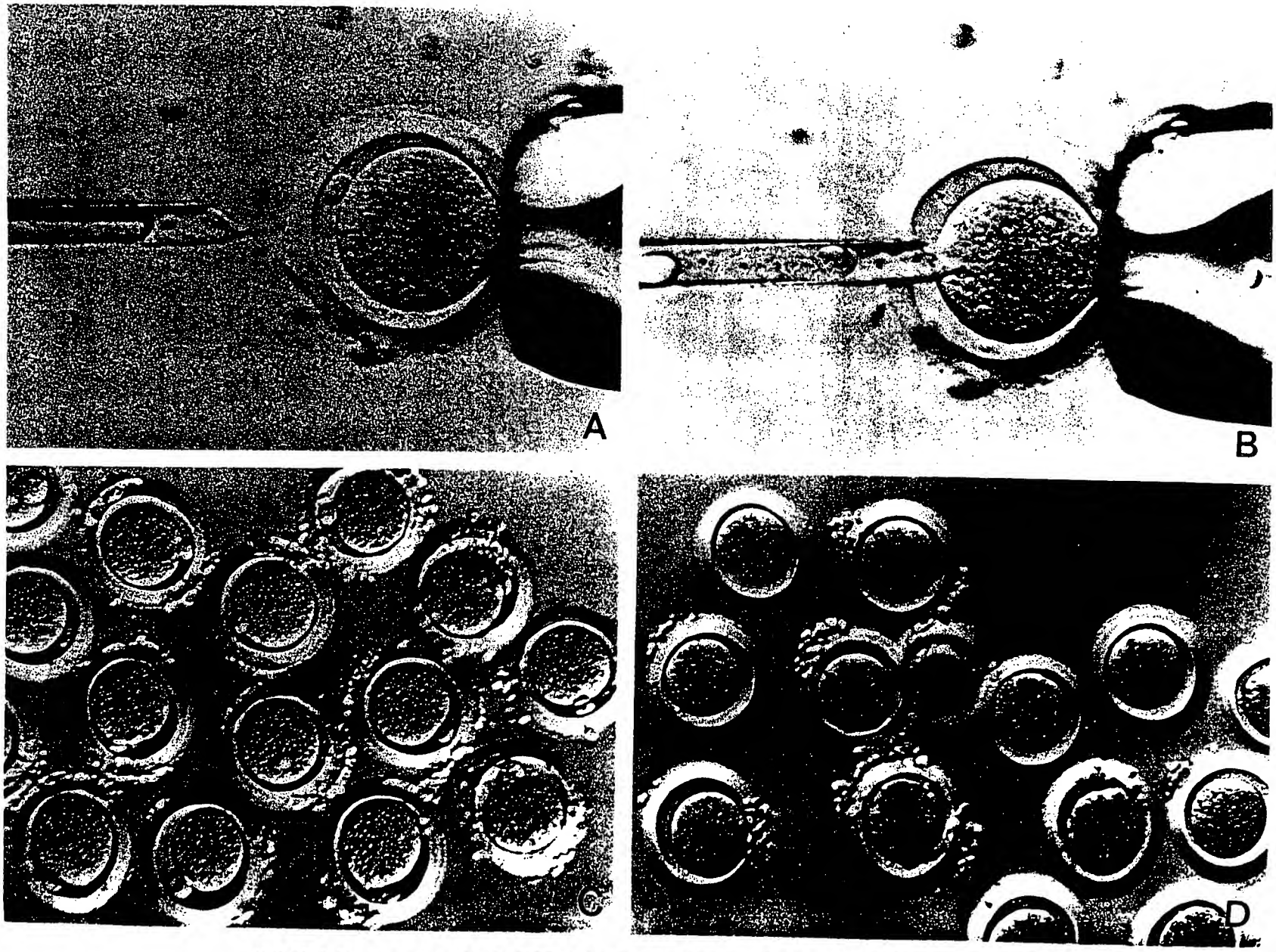


TABLE 1. Effect of DC field strength on oocyte activation.

Field strength (kV/cm)	No. of reps.	No. of oocytes used	Pronuclear oocytes at		Subsequent development (%)	
			7 h (%)	24 h* (%)	2–8-Cell embryos	Morulae and blastocysts
0.0	4	51	7 (14)[a]	17 (33)[a]	5 (10)[a]	0
1.2	4	54	27 (50)[b]	34 (63)[b]	12 (22)[ab]	4 (7)[a]
1.8	4	53	31 (58)[b]	31 (58)[b]	15 (28)[b]	7 (13)[a]
2.4	4	50	43 (86)[c]	45 (90)[c]	36 (72)[c]	14 (28)[b]

[abc]Values with different superscripts within columns differ, $p < 0.05$.
*Including pronuclear and 2–4-cell stages at 24 h.

selected either for transfer into pseudopregnant recipients as reported previously [14] or for further culture to the blastocyst stage.

All activated oocytes were cultured as described above for 4 days to allow time for development into blastocysts. During culture, pronuclear formation (7 h after culture), cleavage division, and development into morulae or blastocysts were recorded.

Embryo Transfer

Depending on the number of embryos available, 12–22 nuclear transferred embryos were placed into the two oviducts of each recipient female. When co-transfer was performed, 12–14 nuclear transferred embryos were placed in one oviduct, and 6 control embryos cultured for 20–22 h were placed in the contralateral oviduct. Cesarean sections were done on Day 29 of pregnancy to identify nuclear transfer progeny in the case of co-transfer, because coat-color markers were not used in this research. Noncultured and nonmanipulated 2–4-cell embryos were similarly transferred to recipients as a positive control.

Evaluation of Enucleation

A simple procedure of chromosomal analysis was used to evaluate the efficiency of enucleation. Because the matured oocytes were at metaphase stage and exact chromosome counting was not necessary, enucleated oocytes were dropped on a glass slide without prior drug or hypotonic treatments. After air-drying on a hot plate for a few minutes, they were placed in fresh fixative (methanol:acetic acid, 3:1) overnight. They were then stained with 5% Giemsa stain (5% of Sigma stock solution in water) for 10 min, rinsed, and air-dried. The oocytes were then evaluated microscopically as enucleated (no chromosomes), partially enucleated (one to several chromosomes), and nonenucleated (with a complete set of chromosomes).

Data Analysis

Activation data were analyzed by one-way ANOVA followed by use of a least-squares multiple comparison test for testing differences among means. Nuclear transfer results were analyzed by chi-square. Birth weight was compared by Student's *t*-test. Treatment differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of Field Strength

When different field strengths of a DC pulse stimulus were applied to activate rabbit oocytes, the 2.4 kV/cm treatment group had the highest ($p < 0.05$) activation rate for all criteria examined (Table 1). The majority (>80%) of activated oocytes with 1 or 2 pronuclei at 7 h for the 2.4 kV/cm treatment group cleaved to 2–4 cells at 24 h. However, less than half of these cleaved in other treatment groups, indicating inadequate activation. Oocytes that had developed only to pronuclear stage by 24 h (retarded) rarely developed to cleavage in any groups examined.

Effect of Extracellular Ions and AC Stimulation

The effects of applying an AC stimulus immediately before the DC pulse and of supplementing the medium with 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$ were tested. As shown in Table 2, the AC stimulus exhibited a positive effect on

TABLE 2. Effect of AC stimulus and exogenous calcium/magnesium on activation and development of rabbit oocytes.

Activation treatment		No. of reps.	Total no. of oocytes	No. and (%) of oocytes activated		
AC	Ca^{2+}/Mg^{2+}			Pronuclear oocytes	2–8-Cell embryos	Morulae or blastocysts
+	+	4	48	37 (77)[a]	34 (71)[a]	20 (42)[a]
+	−	4	58	46 (79)[a]	46 (79)[a]	23 (40)[a]
−	+	4	52	35 (67)[a]	28 (54)[b]	9 (17)[b]
−	−	4	40	29 (73)[a]	22 (55)[b]	11 (28)[ab]

[ab]Values with different superscripts within columns differ, $p < 0.05$.

TABLE 3. Effect of activation medium and cytochalasin (Cyto) B on rabbit oocyte activation.

Activation treatment*	No. of reps.	No. of oocytes used	No. (%) of eggs developed			
			With pronucleus	2–4-Cell stage	Morulae	Blastocysts
Cyto B activation	10	151	92 (61)[a]	90 (60)[a]	79 (52)[a]	58 (38)[a]
Mannitol activation	10	117	70 (60)[a]	69 (59)[a]	49 (42)[a]	30 (26)[b]
Cyto B control	7	89	27 (30)[b]	28 (31)[b]	19 (21)[b]	4 (4)[cd]
Mannitol control	10	127	58 (46)[c]	59 (46)[c]	27 (21)[b]	13 (10)[d]
DPBS control	9	102	34 (34)[b]	35 (35)[b]	9 (8)[c]	1 (1)[c]

[a,b,c,d]Values with different superscripts within columns differ, $p < 0.05$.

*Cyto B activation refers to egg exposure to preincubated M199-FCS with 7.5 μg/ml cytochalasin B for 1 h followed by electric activation (2.4 kV/cm) in 0.3 M Mannitol. Mannitol activation refers to eggs held in M199-FCS for 1 h followed by electrical pulses. Controls were not pulsed electrically. Details are described in the text.

activation, whereas no improvement was observed when calcium and magnesium ions were added to the activation medium (0.3 M M+). However, addition of calcium and magnesium to activation medium was consistently observed to reduce lysis (or loss of blastomeres) during fusion after nuclear transfer manipulation.

Effect of Cytochalasin B

Exposure of oocytes to cytochalasin B for 1 h (the time needed for nuclear transfer manipulation), followed by activation treatment, significantly improved oocyte development into blastocysts compared with oocytes activated without pre-exposure to cytochalasin B (Table 3, 38% vs. 26%, $p < 0.05$). In contrast, pronuclear formation, cleavage division (2–4 cells), or development into morulae was not different ($p > 0.05$) between the two activation treatments. Cytochalasin B exposure for 1 h alone without electric pulses did not cause more activation than the DPBS control ($p > 0.05$). Activation or fusion medium (0.3 M M+) treatment of oocytes for 10 min without electric pulses activated more oocytes than did DPBS treatment for all criteria tested ($p < 0.05$).

Oocyte Enucleation and Nuclear Transfer

Mechanical removal of chromosomes by blind aspiration normally results in either complete enucleation or failure. In this study, the enucleation efficiency of complete chromosomal removal was 77% (n = 111), and that of partial removal was 1%, with the remaining 22% of the oocytes retaining a complete set of chromosomes.

When freshly ovulated metaphase II oocytes were used for enucleation, over 99% of the oocytes contained distinguishable polar bodies. About 5% of them contained two or more polar bodies. Of 491 oocytes used for nuclear transfer, 459 (93%) survived the micromanipulation and fusion procedures, of which 370 (81%) were successfully fused, and 284 (77%) of these fused oocytes developed into 2–4 cells. A majority of the 2–4-cell embryos (275/284) resulting from nuclear transfer appeared to be normal morphologically (Fig. 2A). However, when a subset sample of these (n = 32) were cultured in vitro, only 44% developed to the blastocyst stage (Fig. 2, B and C). This contrasted with 94% blastocyst development of the controls (n = 51, $p < 0.05$).

A total of 243 2–4-cell-stage embryos produced by nuclear transfer (Table 4) were transferred into 15 recipients (with or without co-transfer of control embryos). Eight embryos (3%) developed to term, significantly less than for controls ($p < 0.05$). Among the 15 recipients, 10 received nuclear transferred embryos only (12–22 embryos/recipient), of which 7 were not pregnant, 2 produced 2 dead fetuses (Cesarean section), and 1 produced 4 live young. The progeny may have been from more than one donor morula, on the basis of their appearance (Fig. 2D), since several morulae were pooled to produce individual blastomeres for transfer. The other 5 recipients each received 12–14 nuclear transferred embryos in one oviduct and 6 cultured control embryos in the other. Laparotomy was performed on Day 29 of pregnancy to identify progeny from nuclear transfer. Four of the 5 recipients were pregnant, 2 produced 2 young each from control embryos only, 1 had 1 young (plus 1 absorbed fetus) from nuclear transfers and 3 young from control embryos. Another recipient also had 1 young (plus 1 absorbed fetus) from nuclear transfers and 1 young from control embryos.

When 88 noncultured, nonmicromanipulated 2–4-cell embryos were transferred to 9 recipients, 8 became pregnant and produced 36 young (41%), a rate significantly higher than the 20% survival of control embryos transferred after culture in vitro for 20–22 h ($p < 0.05$, Table 4). Young from nuclear transferred and cultured or noncultured control embryos appeared to be normal and were not different in weight ($p > 0.05$), although young from nuclear transfers tended to weigh more.

DISCUSSION

The rabbit is an inexpensive biologically relevant model for nuclear transfer research for cattle, and perhaps other domestic species [9, 13], particularly because successful nuclear transfer with the embryonic donor cell stage in rabbits parallels these species. Progeny by nuclear transfer of blastomeres from 8-cell [4] and 8–16-cell [5] embryos have

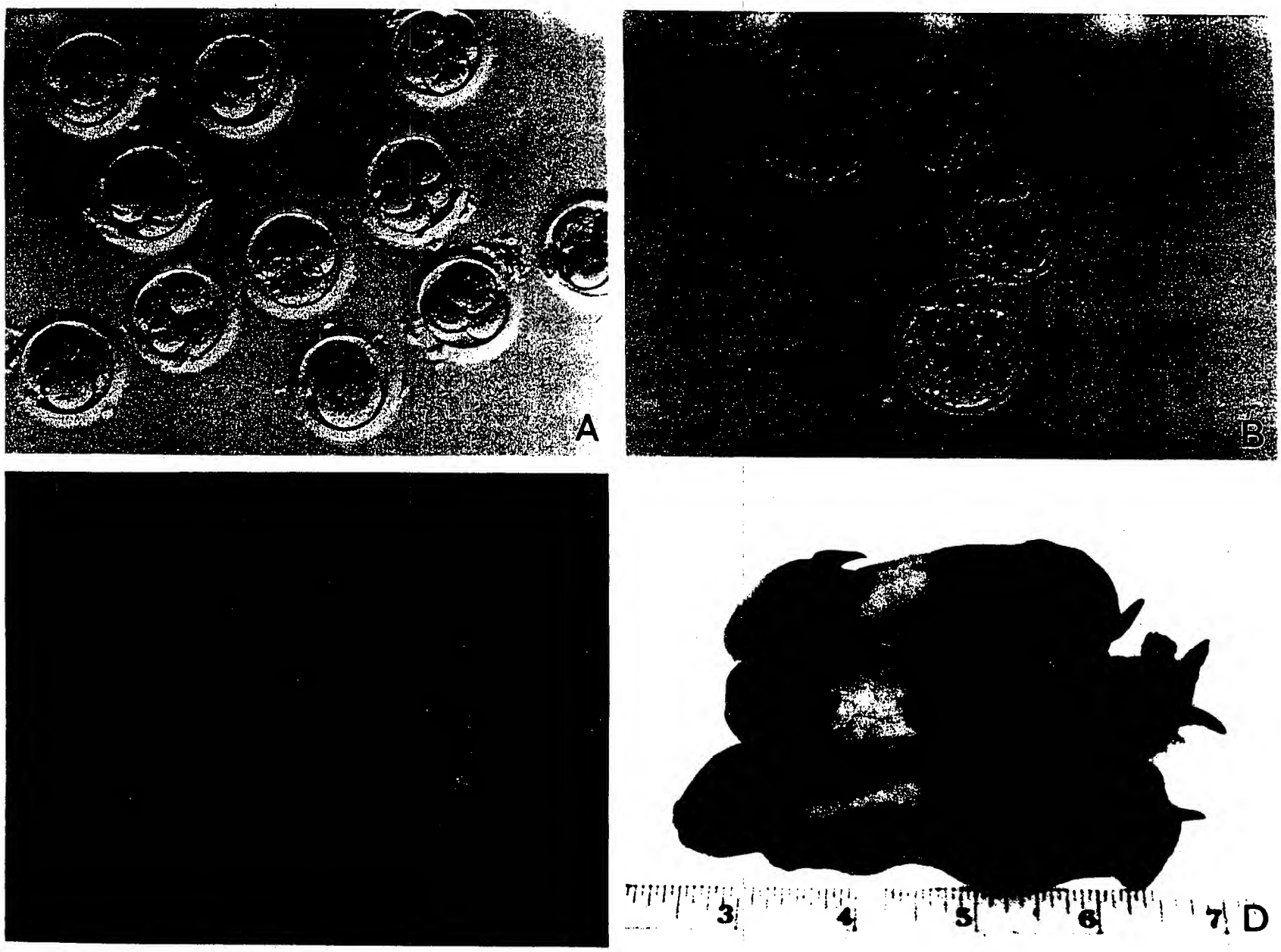


FIG. 2. Embryo development of nuclear transfers: A) A group of embryos produced by nuclear transfer after culture in vitro for 20–22 h. ×175. B) Two hatching blastocysts obtained after nuclear transfer and culture for 4 days. ×280. C) A hatching blastocyst stained with Hoechst 33342 dye containing 153 cells. ×500. D) Progeny obtained from embryos produced by nuclear transfer.

TABLE 4. Survival following transfer of embryos resulting from nuclear transfer and culture versus control eggs, both cultured and noncultured.

Types of embryos	No. of reps.	No. of eggs	No. of recipients		Young (%)
			Total	Pregnant	
Nuclear transfer	8	243	15	4	8 (3)[a]
Cultured (20 h)*	4	30	5	4	6 (20)[b]
Cultured (0 h)	3	88	9	8	36 (41)[c]

[a,b,c]Values with different superscripts differ, $p < 0.05$.
*Cultured 2- to 4-cell embryos cotransferred with nuclear transferred eggs.

been produced in rabbits. Recently, nuclear transfer using fresh [15] or frozen [16] rabbit morulae as nuclear donor cells has resulted in progeny. We report here pregnancy results using rabbit morulae (32–64 cells), produced by culturing 8-cell embryos for about 20–24 h, as nuclear donors, demonstrating that nuclear totipotency remains during this culture period.

Electric pulse-induced activation of rabbit oocytes has been reported previously [4, 5, 17, 18]. The work of Ozil [19], unlike early reports by others [4, 5], indicates that the optimal field strength required for activation probably varies with ion (e.g., calcium and magnesium) concentrations and number of pulses.

Parthenogenetic activation of rabbit oocytes by electric stimulation in mannitol solution with or without calcium and magnesium has been reported previously [4, 5, 12], and a beneficial effect of an AC alignment pulse has been found for fusion [8]. In this report, we tested the effects of supplementing activation medium with calcium and magnesium and of employing an AC pulse immediately before the DC activation pulse. Exogenous calcium and magnesium demonstrated no beneficial effect in our system for activation. This is contrary to the finding of Robl et al. [18] that electric pulse-induced activation of oocytes was caused by influx of exogenous calcium. However, according to Robl (personal communication), it is possible that sufficient amounts of calcium may have diffused into the activation medium from the glass containers used to store the medium. Further study is needed to confirm this. The beneficial effect of the AC pulse on activation is also surprising. During activation treatment, AC-induced oocyte morphological elongation was often observed. This stress to oocytes is a possible explanation of the AC effect on activation. Future research is required to examine whether or not AC stimulus alone may cause oocyte activation.

In another experiment, we tested the effect of nuclear transfer and fusion conditions on the rate of oocyte activation. These conditions included exposure of oocytes in cytochalasin B for 1 h followed by electrical stimulation in mannitol solution. When oocytes were pretreated with cytochalasin B and were activated in mannitol solution plus undergoing the same electrical pulse procedure (Table 3) as was used for nuclear transfer, significantly more activated oocytes developed into blastocysts ($p < 0.05$) compared to oocytes activated similarly without pre-exposure to cytochalasin B. However, pronuclear formation, cleavage division (2–4 cells), and development into morulae were not different ($p > 0.05$) with or without pre-exposure to cytochalasin B. Smith and Wilmut [8] found that incubation of nuclear transferred sheep embryos in cytochalasin B for 1 h after electrical stimulation improved development to morula and blastocyst stages. Collas and Robl [5] confirmed this result in rabbits. The improved development was probably due to a reduced proportion of fragmentation between 16-cell and 64-cell stages when cytochalasin B was used [5]. This may explain our results of higher blastocyst formation in the cytochalasin B activation group, since we did not see increased rates of pronuclear formation and cleavage development when cytochalasin B was used for activation. In addition, cytochalasin B exposure itself without electric pulses did not cause increased activation and development (Table 3).

This experiment (Table 3) also demonstrated that exposing oocytes to activation medium for 10 min increased the rate of activation compared to DPBS control treatment ($p < 0.05$, Table 3). It was observed previously [5] that exposing oocytes to fusion medium for 1 h had a positive effect on activation. This effect may reflect ion movement and osmotic stress to the oocyte following medium change. Also noteworthy is the fact that the activation rate of controls was higher than we reported earlier [12, 13]. The higher rate may have been due to in vitro aging and associated maturation of oocytes in this experiment, as was reported for rabbits by others [4, 5].

The rate of blastomere-oocyte fusion after alignment by AC current was similar to our previous results when mechanical alignment was used [12]. Smith and Wilmut [8] found an improvement in electrofusion when AC current was included, especially for donor cells with a small inner cell mass. Further improvement in activation and fusion may be possible when multiple DC pulses are applied [5, 15].

Lysis during micromanipulation and fusion was about 7%, ranging from 3% to 10% among replicates. Rate of membrane fusion (75%) varied from 60% to 90%. However, there was significant variation among oocyte donors in the development of nuclear transferred embryos. This was also true for activation of oocytes, which is highly correlated with the nuclear transfer result within animals (Yang et al., unpublished observation), indicating the need for the improvement of activation procedures.

Development of nuclear transferred embryos in vitro to the blastocyst stage was 44% when a small number of nuclear transferred embryos were cultured for 4 days. This percentage was much lower ($p < 0.05$) than for control embryos (94%) cultured similarly. The difference is probably due to many factors, such as higher sensitivity of the micromanipulated embryos than intact embryos to an oviductal environment following transfer [14], inadequate activation of the oocyte to support advanced development, and a nuclear donor cell effect.

The efficiency of embryo development to term in this study was about 3%. This efficiency was significantly lower than for cultured (20%) or noncultured (41%) control embryos, but it does demonstrate the nuclear totipotency and the potential of using 32–64-cell embryos cultured from 8 cells as sources for nuclear transfer. The culture period (20–22 h) may be partly responsible for the low efficiency of the nuclear transferred embryos because of the decreased viability of cultured embryos versus noncultured embryos (20% vs. 41%, $p < 0.05$). Polyploidy and inadequate activation of the micromanipulated eggs, however, could contribute to embryonic loss.

Using multiple pulses to induce adequate activation [19] and fusion, postfusion exposure to cytochalasin B, and optimal culture medium may help to improve the efficiency. Collas and Robl [5] obtained higher development when some of these conditions were applied. Transfer of embryos produced by nuclear transfer also should be tested on a larger scale.

ACKNOWLEDGMENTS

The authors thank Dr. George E. Seidel, Jr., for his critical reading of the manuscript; M. Simkin, E. Wilson, L. Belanger, V. Lattone, and C. McCowan for technical assistance; and D. Bevins for typing the manuscript. The authors appreciate the gift of Buserelin by Hoechst-Roussel Agri-Vet Co. and of the BTX fusion chamber by Biotechnologies and Experimental Research, Inc. The authors sincerely acknowledge Dr. James M. Robl and Dr. Philippe Collas of the University of Massachusetts for kindly demonstrating their micromanipulation procedures and their helpful suggestions during the course of this research.

REFERENCES

1. Willadsen SM. Nuclear transplantation in sheep embryos. Nature (Lond) 1986; 320:63–65.
2. Prather RS, Barnes FL, Sims MM, Robl JM, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 1987; 37:859–866.
3. Bondioli KR, Westhusin ME, Looney CR. Production of identical bovine offspring by nuclear transfer. Theriogenology 1990; 33:165–174.
4. Stice SL, Robl JM. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol Reprod 1988; 39:657–664.
5. Collas P, Robl JM. Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. Biol Reprod 1990; 43:877–884.
6. Prather RS, Sims MM, First NL. Nuclear transplantation in early pig embryos. Biol Reprod 1989; 41:414–418.
7. Willadsen SM. Cloning of sheep and cow embryos. Genome 1989; 31:956–962.
8. Smith LC, Wilmut I. Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. Biol Reprod 1989; 40:1027–1035.
9. Robl JM, Stice SL. Prospects for the commercial cloning of animals by nuclear transplantation. Theriogenology 1989; 31:75–84.
10. Kennelly JJ, Foote RH. Superovulatory response of pre- and post-pubertal rabbits to commercially available gonadotrophins. J Reprod Fertil 1965; 9:177–188.
11. Yang X, Chen Y, Chen J, Foote RH. Potential of hypertonic medium treatment for embryo micromanipulation: I. Survival of rabbit embryos in vitro and in vivo following sucrose treatment. Mol Reprod Dev 1990; 27:110–117.
12. Yang X, Zhang L, Kovacs A, Tobback C, Foote RH. Potential of hypertonic medium treatment for embryo micromanipulation: II. Assessment of nuclear transplantation methodology, isolation, subzona insertion and electrofusion of blastomeres. Mol Reprod Dev 1990; 27:118–129.
13. Yang X. Embryo cloning by nuclear transfer in cattle and rabbits. Int Embryo Transfer Newsletter 1991; 9:10–22.
14. Yang X, Foote RH. Survival of bisected rabbit morulae transferred to synchronous and asynchronous recipients. Mol Reprod Dev 1990; 26:6–11.
15. Collas P, Robl JM. Development of rabbit nuclear transplant embryos from morula and blastocyst stage donor nuclei. Theriogenology 1991; 35:190.
16. Heyman Y, Chesne P, Renard JP. Reprogrammation complete de noyaux embryonnaires congeles, apres transfer nucleaire chez le lapin. CR Acad Sci Paris 1990; 311:321–326.
17. Onodera M, Tsunoda Y. Parthenogenetic activation of mouse and rabbit eggs by electric stimulation in vitro. Gamete Res 1989; 22:277–283.
18. Robl JM, Collas P, Fissore R, Dobrinsky JR. Electrically induced fusion and activation in nuclear transplant embryos. In: Chang D, Chaffy BM, Saunders JA, Sowers AE (eds.), Handbook of Electroporation and Electrofusion. San Diego CA: Academic Press; 1991.
19. Ozil JP. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. Development 1990; 109:117–127.

Journal of Nutrition; Dec 1993; 123, 12; Research Library
152

Nutrient Metabolism

Feeding Status Affects Glucose Metabolism in Exercising Horses[1,2]

LAURIE LAWRENCE,[3] L. VINCENT SODERHOLM, ANDREW ROBERTS, JANICE WILLIAMS AND HAROLD HINTZ

Department of Clinical Science, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

ABSTRACT Four standardbred horses were used in a Latin square design experiment to evaluate the effects of feeding status on metabolic response to exercise. Horses were deprived of food overnight and then fed 0 (control condition), 1, 2 or 3 kg of corn grain approximately 2.5–3 h before exercise. The exercise test consisted of a warm-up phase (heart rate mean = 144 beats/min) followed by 800 m of walking and then a high intensity work bout for 1600 m (heart rate mean = 206 beats/min). All tests were conducted on a high speed equine treadmill on which the horses had been previously conditioned. During exercise, the control treatment resulted in steady plasma glucose concentrations, whereas all other treatments (1, 2 or 3 kg corn) caused a decline in plasma glucose. Liver glycogen concentration was decreased by exercise in the horses only when they received the control treatment. Plasma lactate concentration was increased by exercise but was not affected by feeding state. Plasma free fatty acid concentrations were highest when the horses received the control treatment. In addition, when horses received the control treatment, plasma free fatty acid concentrations decreased during the warm-up and high intensity phases of the test and increased during the walk phase. Free fatty acid concentrations changed in the horses receiving 1, 2 or 3 kg of corn grain only during the high intensity phase of the exercise test. Feeding status affects the metabolic response of horses to exercise thus further studies are necessary to evaluate the effect of feeding status on exercise performance. J. Nutr. 123: 2152–2157, 1993.

INDEXING KEY WORDS:

- *horses* • *exercise*
- *glucose* • *feeding status*

In recent years, there has been increasing interest in the nutrition of both human and equine athletes. Several studies have examined the effect of diet composition on substrate availability and energy metabolism in exercising horses (Hambleton et al. 1980, Miller-Graber et al. 1991, Oldham et al. 1990). However, no studies have evaluated how feeding state affects substrate availability or utilization in exercising horses. In rats, short term food deprivation seems to improve exercise performance, presumably by increasing the utilization of fat and sparing carbohydrate stores (Dohm et al. 1983). However, in humans, performance is impaired when a work bout is undertaken after an extended fast (24–36 h) compared with the post-absorptive state (4–12 h after a meal) (Gleeson et al. 1988, Zinker et al. 1990). In humans, fasting may negatively affect performance by decreasing carbohydrate availability because fasting has been reported to decrease liver glycogen stores (Hultman 1989).

Changing carbohydrate status by consuming food just before exercise has produced conflicting results in exercising humans. Glucose or starch intake 1 to 4 h before exercise will increase the oxidation of carbohydrate (Ahlborg and Felig 1977, Sherman et al. 1991, Willcutts et al. 1988) and can result in a decrease in blood glucose concentration (Costill et al. 1977). Some authors have suggested that these responses may have a detrimental effect on performance (Costill 1985, Williams 1989), but other studies have found positive effects of carbohydrate intake just before exercise (Sherman et al. 1991, Wright et al. 1991).

Following a carbohydrate meal, glucose and insulin responses in horses are qualitatively similar to those in humans, although peak absorptive glucose and insulin concentrations in horses occur somewhat later (2.5–3 h post-feeding) than in humans (Stull and Rodiek 1988). It was hypothesized that horses exercised in the fed state would experience the sharp

[1]Supported by the Harry M. Zweig Memorial Fund for Equine Research.

[2]The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

[3]To whom correspondence should be addressed at: 905 W. P. Garrigus Bldg., University of Kentucky, Lexington, KY 40546.

0022-3166/93 $3.00
Manuscript received 3 November 1992. Initial review completed 11 March 1993. Revision accepted 14 July 1993.

decline in blood glucose observed in humans. It was the purpose of this study to evaluate metabolic responses in horses exercised 2.5–3 h following a carbohydrate meal.

MATERIALS AND METHODS

Horses. Two standardbred mares and two standardbred geldings (aged 3 to 8 y; 443 ± 25.9 kg, mean ± SD) owned by Cornell University were used in the experiment. For 6 wk before the onset and during the 6 wk of the study, the horses were regularly exercised on a motorized treadmill in an environmentally controlled building. All exercise periods were conducted between 0800 and 1300 h. During the study, the horses were individually housed in box stalls and received two meals per day at 0630 and 1530 h. Initially, each horse received a ration of hay (IFN 1-04-884) and a commercial concentrate (Agway, Ithaca, NY) in amounts adequate to maintain body weight. Two weeks before the 6-wk study period, the commercial concentrate provided in the morning feedings was gradually replaced with corn grain (IFN 4-02-935). The experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee at Cornell University.

Treatments. Treatments were assigned in a 4 × 4 Latin square design so that each horse completed an exercise test after receiving 0 (control treatment), 1, 2 and 3 kg of whole corn. On the day before each exercise test, the horses received their regular diet at 0630 and 1530 h. At 1800 h, any uneaten food (hay or concentrate) was removed. Horses had free access to water at all times before the exercise test. On the morning of the test (0600 h), horses received only the test meal.

Exercise test. All exercise tests were conducted between 0830 and 1300 h in the same facility used for the regular exercise bouts. The exercise test was conducted 2.5–3 h after the test meal was offered. When horses received the control treatment they were not fed the morning of the exercise test, therefore 15–18 h elapsed between the time food was removed the previous evening and when those horses performed the exercise test. The exercise test consisted of a warm-up phase, a walking recovery and a high intensity phase (**Fig. 1**). In the warm-up phase, the horses exercised at 6.0 m/s on a flat treadmill surface for 1600 m. The horses were then allowed to walk at 1.9 m/s for 800 m. During the second half of the walk phase, the treadmill grade was increased to 2%. The treadmill speed was then increased to 11 m/s over a distance of 400 m and the horses completed 1600 m at 11 m/s on the 2% grade. The total test covered 4400 m and lasted ~15 min. Heart rate averaged 144 beats/min during the last minute of the warm-up phase and 206 beats/min in the last minute of the high intensity phase. The exercise test performed in this study was intense (heart rate >200 bpm), but was not maximal. Three horses galloped during the high intensity phase and one horse paced. All horses performed a trot or pace during the warm-up phase. Exercise tests were performed at least 8–10 d apart. Horses were not exercised on the day preceding the exercise test.

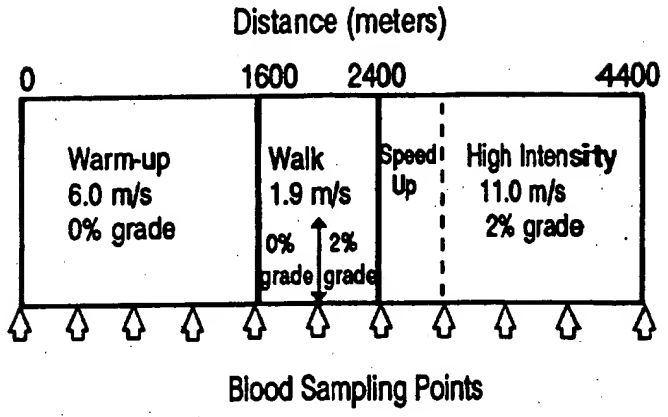


FIGURE 1 Schematic representation of the exercise test performed by the horses on a high speed equine treadmill. Heart rate averaged 144 beats/min during the warm-up phase and increased to 206 beats/min at the end of the high intensity phase.

Sample collection and analysis. One hour before exercise, an indwelling catheter was inserted in the left jugular vein. Before catheterization, the area over the insertion site was clipped, scrubbed with an antiseptic soap and swabbed with alcohol. Following insertion, the catheter was connected to a 40-cm extension set and sutured in place. The extension set and catheter were flushed as needed with sterile, physiologic saline.

Blood samples were collected before the exercise test (0 m) and at 400-m intervals during the test (Fig. 1). Blood for glucose and lactate determinations was placed in tubes containing sodium fluoride and potassium oxalate. Blood for free fatty acid (FFA) and insulin determinations was placed in tubes containing EDTA. Plasma lactate concentrations were determined using an automated lactate analyzer (YSI, Yellow Springs, OH). Plasma glucose and FFA concentrations were determined by enzymatic methods using commercially available reagents (Sigma Chemical, St. Louis, MO, catalogue no. 510A and Wako Chemicals U.S.A., Richmond, VA, catalogue no. 99075401, respectively). Plasma insulin was determined using an RIA previously validated for horses (Reimers et al. 1982).

Muscle biopsy samples were obtained from the middle gluteal muscle before and after exercise for determination of glycogen content. Before the exercise test, the area over the right or left middle

gluteal muscle was clipped and scrubbed using an antiseptic soap. Following local anesthesia, a stab incision was made, and a biopsy was obtained using a biopsy needle with a 6-mm diameter (DePuy, Warsaw, IN). Liver biopsy samples were also obtained under local anaesthesia before and after exercise. After the area over the right 12th intercostal space was clipped and scrubbed, a biopsy needle (Tru-cut Biopsy Needle, Travenol Labs, Deerfield, IL) was inserted through a stab incision and a biopsy of the liver was obtained. Difficulty obtaining a post-exercise liver biopsy was encountered in some horses because of excess abdominal movement associated with increased respiration. Muscle and liver biopsies were immediately frozen in liquid nitrogen and stored at –40°C until analyzed for glycogen content using the method of Passonneau and Lauderdale (1974). Values are expressed on a wet weight basis and represent the mean of duplicate (liver) or quadruplicate analyses (muscle). In several cases, the coefficient of variation for glycogen content of a muscle biopsy exceeded 10%. If sufficient tissue was not available for further analysis, that value was omitted from the data set. Reliable measurements of glycogen content were obtained on fourteen paired liver samples (before and after exercise) and eleven paired muscle samples.

Statistical analysis. Dietary treatment and exercise effects on plasma glucose, insulin, lactate and FFA were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). The data were analyzed with the following model:

$$f_{ijk}(e) = \mu + \gamma_i + \zeta_j + \delta_k + \beta_k e + \epsilon$$

where, μ = the overall average; γ = the effect due to period; i = 1, 2, 3, 4; ζ = the effect due to horse; j = 1, 2, 3, 4; δ = the effect due to treatment; k = 1, 2, 3, 4; and β = the slope of the line due to exercise.

An effect of exercise was considered significant when the slope of the line was different from 0 ($P < 0.05$). When the slope of more than one line (treatment) was different from 0 ($P < 0.05$), differences between slopes were analyzed using a t test. Because the exercise test consisted of three distinct phases (warm-up, walk and high intensity exercise) the data from each phase were analyzed separately. To identify differences between treatments at specific time points, an ANOVA using horse, period and treatment as the main effects was used and means were separated with a least significant difference test.

For statistical analysis of the glycogen data, all fed treatments (1, 2 or 3 kg corn) were grouped together. The effect of exercise on glycogen concentration was examined in each group (fed or control) using a Student's t test for paired samples. Values of $P < 0.05$ were considered significant for all analyses.

RESULTS

The effect of treatment on the plasma glucose response to exercise is shown in **Figure 2.** When the horses received 3 kg of corn grain 2.5–3 h before exercise, initial plasma glucose concentrations were higher than when the horses received the control treatment ($P < 0.05$). When the horses received 1 or 2 kg of corn, pre-exercise plasma glucose concentrations were intermediate to the control and 3-kg treatments. During the exercise test, plasma glucose concentrations remained relatively stable when horses received the control treatment, but decreased markedly during the warm-up and walk phases of the exercise test when the horses received each of the other treatments ($P < 0.05$). During the high intensity phase of the test, there were no treatment differences for plasma glucose concentrations. Changes in plasma insulin concentrations closely resembled the changes in plasma glucose (**Table 1**). During exercise, plasma insulin concentrations declined in each of the fed treatments (1, 2 and 3 kg of corn) but remained relatively stable in the control condition. In the control condition, insulin levels declined slightly during the warm-up and walk phases and reached the lowest level at 2400 m (end of the walk phase).

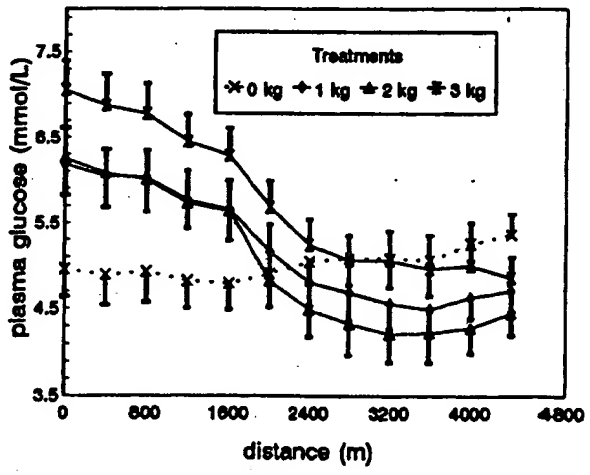


FIGURE 2 Plasma glucose concentrations in exercising horses receiving 0, 1, 2 or 3 kg of corn grain 2.5–3 h before exercise. Each value is the least squares mean ± SE of four observations. During the first 1600 m, the horses performed a warm-up (6.0 m/s) followed by 800 m at a walk (1.9 m/s). The horses galloped or paced (one horse) at 11 m/s on a 2% grade for the remainder of the test. At the initiation of exercise (0 m) plasma glucose concentration was higher when horses were fed 3 kg of corn than when they were not fed (control treatment/0 kg corn) before exercise ($P < 0.05$). Exercise resulted in a decrease in plasma glucose concentrations when horses received 1, 2 or 3 kg of corn before exercise ($P < 0.05$) but not when they received the control treatment. Plasma glucose responses during exercise when horses were fed 1, 2 or 3 kg of corn before exercise did not differ ($P < 0.05$).

TABLE 1

Plasma insulin concentrations during the exercise test when horses received 0, 1, 2 or 3 kg of corn 2.5–3 h before exercise[1,2]

Distance	Treatment (*kg of corn fed*) 0	1	2	3	SE
m		*pmol/L*			
0	123.3	191.7	288.3	311.7	81.7
400	88.3	195.0	266.7	238.3	61.7
800	88.3	228.3	296.7	231.7	51.7
1200	68.3	230.0	235.0	273.3	51.7
1600	88.3	160.0	213.3	201.7	70.0
2000	93.3	128.3	135.0	183.3	50.0
2400	45.0	100.0	130.0	170.0	31.7
2800	85.0	118.3	136.7	166.7	35.0
3200	103.3	120.0	118.3	180.0	31.7
3600	108.3	126.7	118.3	120.0	25.0
4000	88.3	85.0	143.3	140.0	30.0
4400	83.3	100.0	140.0	106.7	28.3

[1]The exercise test consisted of the following: warm-up phase = 0 to 1600 m, walk phase = 1600 to 2400 m, high intensity phase = 2400 to 4400 m.

[2]Each value represents the least squares means of four observations. During the exercise test plasma insulin concentrations decreased when horses were fed 1, 2 or 3 kg corn before exercise ($P < 0.05$).

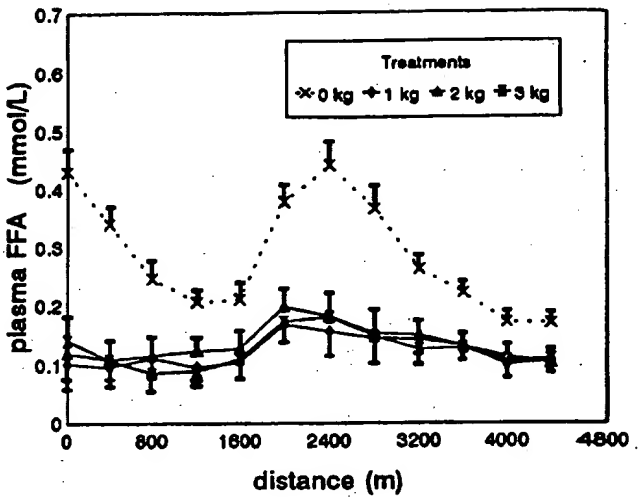


FIGURE 3 Plasma free fatty acid (FFA) concentrations in exercising horses receiving 0, 1, 2 or 3 kg of corn grain 2.5–3 h before exercise. During the first 1600 m, the horses performed a warm-up (6.0 m/s) followed by 800 m at a walk (1.9 m/s). The horses galloped or paced (one horse) at 11 m/s on a 2% grade for the remainder of the test (high intensity phase). Values are the least squares means ± SE of four observations. At the onset of exercise (0 m) plasma FAA concentrations were higher when horses received the control treatment (0 kg corn) than when they received 1, 2 or 3 kg of corn ($P < 0.05$). When horses received the control treatment, plasma FFA concentrations decreased during the warm-up, increased during the walk and decreased during the high intensity phase ($P < 0.05$). When horses received 1, 2 or 3 kg of corn before exercise, plasma FFA concentrations did not change during the warm-up or walk but decreased during the high intensity phase ($P < 0.05$).

As noted previously, adequate samples for muscle and liver glycogen determinations were not obtained on all horses. Table 2 shows the effect of exercise on glycogen concentration when the values for all fed (1, 2 and 3 kg corn) groups are combined. Liver glycogen concentration decreased as a result of exercise in the control treatment ($P < 0.05$) but not in the fed treatment. There was no treatment effect on muscle glycogen ($P > 0.1$). Plasma lactate concentrations increased with exercise ($P < 0.05$) but were not affected by feeding state (Table 3).

The effect of the exercise test on FFA concentrations is shown in Figure 3. Free fatty acid concentrations were consistently higher and were more responsive to exercise when horses received the control treatment compared with any of the fed treatments. When horses received the control treatment, FFA concentrations decreased during the warm-up phase of the test ($P < 0.05$), increased during the walk phase ($P < 0.05$) and then decreased again during the high intensity phase ($P < 0.05$). Conversely, when

TABLE 2

Muscle and liver glycogen concentrations in control and fed horses performing an exercise test[1,2,3]

		Liver glycogen			Muscle glycogen	
		Pre-exercise	Post-exercise		Pre-exercise	Post-exercise
				μmol/g wet tissue		
Fed	(*n* = 11)	297.7 ± 30.2	313.5 ± 30.6	(*n* = 8)	106.5 ± 9.3	105.1 ± 12.9
Control	(*n* = 4)	254.1 ± 17.3	225.6 ± 19.5*	(*n* = 3)	123.1 ± 11.1	121.1 ± 6.9

[1]Values are means ± SE. *Different from pre-exercise ($P < 0.05$) using Student's *t* test for paired samples.

[2]Fed, horses received 1, 2 or 3 kg of corn grain 2.5–3 h before exercise.

[3]Control, horses exercised after receiving no food for 15–18 h.

TABLE 3

Plasma lactate concentrations during the exercise test when horses received 0, 1, 2 or 3 kg of corn 2.5–3 h before exercise[1,2]

Distance	Treatment (*kg of corn fed*) 0	1	2	3	SE
m	*mmol/L*				
0	0.8	0.8	0.8	0.9	0.04
400	1.7	1.6	1.4	1.4	0.04
800	2.1	1.7	1.4	1.5	0.62
1200	1.8	1.4	1.3	1.4	0.50
1600	1.6	1.3	1.2	1.2	0.40
2000	1.1	0.9	0.9	0.8	0.22
2400	0.9	0.8	0.8	0.7	0.22
2800	1.7	1.7	1.4	1.7	0.22
3200	5.9	6.1	5.2	5.7	0.58
3600	9.5	9.0	8.9	8.6	0.58
4000	11.8	11.0	11.5	10.8	1.44
4400	13.6	12.8	14.0	12.5	2.00

[1]The exercise test consisted of the following: warm-up phase = 0 to 1600 m, walk phase = 1600 to 2400 m, high intensity phase = 2400 to 4400 m.

[2]Values are least squares means of four observations. Exercise resulted in an increase in plasma lactate concentration in each treatment ($P < 0.05$). There were no differences between treatments ($P > 0.05$).

horses received a pre-exercise meal of 1, 2 or 3 kg of corn, FFA concentrations were relatively low and did not change during the warm-up or walk phase of the exercise test. A decline in FFA concentrations did occur during the high intensity phase of the test ($P < 0.05$) when the horses received a pre-exercise meal, but the size of the meal (1, 2 or 3 kg) did not affect the response.

DISCUSSION

In athletes, the objective of most dietary manipulations is a modification of substrate availability and use in working muscle. Although consumption of a carbohydrate meal has a direct effect on substrate availability by increasing blood glucose concentration, a more important effect of feeding may be exerted through changes in hormone concentrations. In this study, when horses were fed 1, 2 or 3 kg whole corn grain, 2.5–3 h before exercise, there was a sharp decline in blood glucose concentration during the exercise bout, compared with when the horses were not fed for 15–18 h before exercise. The decline in plasma glucose concentration may have resulted from an enhanced uptake of glucose by muscle under the influence of insulin. In humans, the elevated insulin concentrations after eating result in enhanced glucose uptake during exercise (Ahlborg and Felig 1977). It is also possible that the decrease in plasma glucose may have resulted from an impaired ability of hepatic glycogenolysis to maintain plasma glucose concentrations. When the horses received a pre-exercise meal, hepatic glycogen was not affected by the exercise test. However, in the horses that were not fed before exercise, hepatic glycogen concentration was decreased after exercise.

Although it is possible that feeding the horses 2.5–3 h before exercise may have resulted in greater blood glucose utilization during exercise, there was not a concomitant effect on lactate production. Plasma lactate concentration was not affected by feeding state in this study. When humans exercise at 50–75% of maximal oxygen uptake (VO_{2max}), a pre-exercise meal may or may not increase lactate production (Maughan and Gleeson 1988, Sherman et al. 1991, Zinker et al. 1990). Gleeson et al. (1988) observed that ingesting a meal 4 h before high intensity exercise (100% VO_{2max}) resulted in higher blood lactate concentrations at the end of exercise. However, in that study, individuals that consumed carbohydrate before exercising were able to exercise longer than those who had fasted before exercise; therefore, the higher lactate levels may have been a consequence of a greater work effort rather than increased glucose availability. Because the accumulation of lactate and hydrogen ions is associated with fatigue during high intensity exercise, dietary manipulations that enhance lactate production could have a detrimental effect on performance. The data presented here suggest that ingesting a meal 2–3 h before an intense exercise bout will not enhance anaerobic glycolysis or increase lactate production in horses.

In humans, fasting and eating practices before exercise have produced conflicting results on endurance-type performance (Costill et al. 1977, Maughan and Gleeson 1988, Wright et al. 1991). Lewis (1982) recommends that horses undertaking long duration exercise (endurance racing) should not be given a high concentrate meal after the evening before the event. The observations of this study may support that recommendation in that feeding the horses 2.5–3 h before exercise resulted in a sharp decline in blood glucose during exercise and low FFA availability. In addition, when the horses received no pre-exercise meal, they were able to use hepatic glycogenolysis to maintain glucose homeostasis to a greater extent than when they were fed before exercise. Because this study was not specifically designed to investigate endurance exercise, it is difficult to draw conclusions as to the effects of a high carbohydrate meal on the metabolism or performance in horses involved in endurance activities.

When humans exercise in the fed state, proportionally more carbohydrate and less fat will be catabolized than when the exercise is performed in the

fasting state, resulting in a higher respiratory exchange ratio (Willcutts et al. 1988, Wright et al. 1991). Although we did not measure respiratory exchange ratio, it was apparent that horses receiving 1, 2 or 3 kg of corn before exercise had lower concentrations of FFA available for oxidation. Costill et al. (1977) demonstrated that FFA availability is an important determinant of the amount of fat that will be oxidized during exercise. Lipolysis in adipose tissue is enhanced by glucagon and catecholemines and suppressed by insulin. It is possible that FFA mobilization from adipose tissue was suppressed by the elevated insulin in the fed horses, resulting in lower plasma FFA concentrations. Plasma FFA concentrations increased during the walk period after the warm-up in the horses while receiving the control treatment, and the highest plasma FFA concentration coincided with the lowest insulin concentration.

Some researchers have observed no changes in plasma FFA concentrations in intensely exercised horses and have concluded that plasma FFA are not used during this type of exercise (Pagan et al. 1987). The observations of previous studies may have resulted because horses were exercised without a warm-up, in a fed state. The contention that FFA can be used during intense exercise is supported by the work of Snow et al. (1983). In that study, racing resulted in a change in the composition of the plasma FFA pool even though the concentration of total FFA was relatively unchanged. Our observation that FFA concentrations decreased during high intensity work also supports this contention. Clearly, conclusions about FFA use cannot be based on changes in plasma concentrations alone; further elucidation of this area awaits studies on FFA turnover.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the able assistance of Jennifer Henry, Amy Johnson, Tracy Fisher, Pam Mueller, Renate Rehder and Kathy Petersson.

LITERATURE CITED

Ahlborg, G. & Felig, P. (1977) Substrate utilization during prolonged exercise preceded by ingestion of glucose. Am. J. Physiol. 233: E 188–194.

Costill, D. L. (1985) Carbohydrate nutrition before, during, and after exercise. Fed. Proc. 44: 364–368.

Costill, D. L., Coyle, E., Dalsky, D., Evans, G., Fink, W. & Hoopes, D. (1977) Effects of elevated plasma FFA and insulin on muscle glycogen usage during exercise. J. Appl. Physiol. 43: 695–699.

Dohm, G. L., Tapscott, E. B., Barakat, H. A. & Kasparek, G. J. (1983) Influence of fasting on glycogen depletion in rats during exercise. J. Appl. Physiol. 55: 830–833.

Gleeson, M., Greenhaff, P. & Maughan, R. J. (1988) Influence of a 24-h fast on high intensity cycle exercise performance in man. Eur. J. Appl. Physiol. 57: 653–659.

Hambleton, P. L., Slade, L. M., Hamar, D. W. & Kienholz, E. D. (1980) Dietary fat and exercise conditioning effect on metabolic parameters in the horse. J. Anim. Sci. 51: 1330–1339.

Hultman, E. (1989) Nutritional effects on work performance. Am. J. Clin. Nutr. 49: 949–957.

Lewis, L. D. (1982) Feeding and Care of the Horse. Lea and Febiger, Philadelphia, PA.

Maughan, R. J. & Gleeson, M. (1988) Influence of a 36-h fast followed by refeeding with glucose, glycerol or placebo on metabolism and performance during prolonged exercise in man. Eur. J. Appl. Physiol. 57: 570–576.

Miller-Graber, P. A., Lawrence, L. M., Foreman, J. H., Bump, K. D., Fisher, M. G. & Kurcz, E. V. (1991) Dietary protein level and energy metabolism during treadmill exercise in horses. J. Nutr. 121: 1462–1469.

Oldham, S. L., Potter, G. D., Evans, J. W., Smith, S. B., Taylor, T. S. & Barnes, W. S. (1990) Storage and mobilization of muscle glycogen in exercising horses fed a fat-supplemented diet. J. Equine Vet. Sci. 10: 353.

Pagan, J. D., Essen-Gustavsson, B., Lindholm, A. & Thornton, J. (1987) The effect of dietary energy source on blood metabolites in Standardbred horses during exercise, pp. 425–430. Proceedings of the 10th Equine Nutrition and Physiology Symposium. Ft. Collins, CO.

Passonneau, J. R. & Lauderdale, V. R. (1974) A comparison of three methods of glycogen measurement in tissues. Anal. Biochem. 60: 405–412.

Reimers, T. J., Cowan, R. G., McCann, J. P. & Ross, M. W. (1982) Validation of rapid solid-phase radioimmunoassay for canine, bovine, and equine insulin. Am. J. Vet. Res. 43: 1274.

Sherman, W. M., Peden, M. C. & Wright, D. (1991) Carbohydrate feedings 1 h before exercise improves cycling performance. Am. J. Clin. Nutr. 54: 866–870.

Snow, D. H., Fixter, L. M., Kerr, M. G. & Cutmore, C.M.M. (1983) Alterations in composition of venous plasma FFA pool during prolonged and sprint exercise in the horse. In: Biochemistry of Exercise (Knuttgen, H. G., Vogel, J. A. & Poortmans, J., eds.), pp. 336–342. Human Kinetics Publishers, Champaign, IL.

Stull, C. L. & Rodiek, A. V. (1988) Responses of blood glucose, insulin and cortisol concentrations to common equine diets. J. Nutr. 118: 206–213.

Willcutts, K. F., Wilcox, A. R. & Grunewakl, K. K. (1988) Energy metabolism during exercise at different time intervals following a meal. Int. J. Sports Med. 9: 240–243.

Williams, L. (1989) Diet and endurance fitness. Am. J. Clin. Nutr. 49: 1077–1083.

Wright, D. A., Sherman, W. M. & Dernback, A. R. (1991) Carbohydrate feedings before, during, or in combination improve cycling endurance performance. J. Appl. Physiol. 71: 1082–1088.

Zinker, B. A., Britz, K. & Brooks, G. A. (1990) Effects of a 36-hour fast on human endurance and substrate utilization. J. Appl. Physiol. 69: 1849–1855.

Journal of Nutrition; Jan 1993; 123, 1; Research Library

Nutrient Metabolism

Energy Restriction Reduces Metabolic Rate in Adult Male Fisher-344 Rats[1]

DIANA M. GONZALES-PACHECO, WILLIAM C. BUSS,* KATHLEEN M. KOEHLER, WILLIAM F. WOODSIDE[2] AND SEYMOUR S. ALPERT†[3]

Department of Health Promotion, Physical Education, and Leisure Programs, *Department of Pharmacology and †Department of Physics and Astronomy, University of New Mexico, Albuquerque, NM 87131

ABSTRACT Energy restriction, without malnutrition, prolongs the maximum life span of laboratory rodents. A reduction in metabolic rate has been proposed as a potential mechanism for increased longevity. The present study examines changes in metabolic rate of adult rats after a 6-wk period of energy restriction. Two groups (n = 6) of 6-mo-old male Fisher-344 rats were studied. Restricted rats were pair-fed a diet equivalent in vitamins and minerals but restricted to 60% of energy consumed by rats eating ad libitum. Average and basal metabolic rates were measured by direct calorimetry over a 24-h period without food. Fat mass and lean body mass were determined by NMR spectroscopy. After 6 wk of restriction, when expressed per kilogram of lean body mass the average metabolic rate was reduced by 14% and basal metabolic rate by 12% compared with the ad libitum diet rats ($P \leq 0.01$). Reduction of metabolic rate did not seem to be a transient effect of chronic energy restriction in mature rats. J. Nutr. 123: 90–97, 1993.

INDEXING KEY WORDS:

- *energy restriction* • *Fisher-344 male rats*
- *energy balance* • *direct calorimetry*
- *body composition*

Energy restriction, without malnutrition, has been shown to prolong the maximum life span of laboratory rodents and delay the occurrence of age-related changes in physiological function and age-associated disease processes (Masoro 1984). The action of energy restriction is related to reduction in energy rather than to reduction in specific nutrients such as fat, protein or minerals. Initiation of energy restriction in early adult life has been demonstrated to be as effective as initiation of energy restriction soon after weaning (Yu et al. 1982). The mechanism of energy restriction on the aging processes is not known, but reduction of metabolic rate per unit body mass has been proposed (Forsum et al. 1981, Harman 1981, Sacher 1977, Sacher and Duffy 1979).

A number of studies over the past 40 y have investigated the relationship among energy restriction, longevity and energy metabolism, and the results are conflicting. Reported effects of energy restriction on metabolic rate range from no change (Duffy et al. 1989, McCarter et al. 1985), to a transient decrease (McCarter and McGee 1989, Mohan and Narasinga Rao 1983), to sustained decreases (Forsum et al. 1981, Hill et al. 1985 and 1988). Differences in the study designs make it difficult to compare results. Variables that may account for discrepancies include the age of animals studied, animal strain, duration and level of energy restriction, feeding period (light vs. dark), instrument methodology, data collection methods and data normalization.

An acute adaptation to energy restriction is a decreased metabolic rate (Weindruch and Walford 1988). A limited number of studies have examined the effects of chronic energy restriction on metabolic rate (Duffy et al. 1989, Hill et al. 1988, McCarter and McGee 1989, McCarter et al. 1985). Results reported by McCarter et al. (1985) led Masoro (1988) to conclude that metabolic rate per gram lean body mass is not affected by chronic energy restriction and, therefore, does not explain the relationship between energy restriction and longevity. However, Weindruch and Walford (1988) argued that the limited available data do not provide adequate grounds on which to abandon the hypothesis of decreased metabolic rate as an explanation of the effects of energy restriction on longevity. It seems likely that the responses to energy restriction result from a number

[1]This work was conducted in partial fulfillment of a Master of Science degree by Diana M. Gonzales-Pacheco.

[2]We deeply regret the unexpected death of Dr. William F. Woodside on February 5, 1992.

[3]To whom correspondence should be addressed.

of factors, including possible changes in metabolic rate. Therefore, further investigation into the effects of energy restriction on metabolic rate is warranted to clarify its contributing role.

The primary aim of this investigation was to determine the relationship between dietary restriction to a level of 60% of ad libitum intake and average metabolic rate of mature adult rats. McCarter and McGee (1989) reported that the initial reduction of metabolic rate in response to energy restriction was transient and was lost within a few weeks; therefore, a 6-wk period of treatment was chosen to permit observation of effects after the reported transient phase. Heat loss measurements were made using direct calorimetry.

MATERIALS AND METHODS

Male Fisher 344/NHsd rats (n = 17) were purchased from Harlan Sprague Dawley, (Indianapolis, IN) at 6 mo of age. The rats were raised under barrier conditions, given free access to nonpurified diet (NIH 31, Teklad, Madison, WI), and housed in groups of 1–6 before our receiving them. The rats were received in three separate shipments. Animals were housed singly in hanging stainless steel cages at the University of New Mexico School of Medicine Animal Resource Facility and maintained on a 12-h light:dark cycle (light: 0600–1800 h). Temperature was maintained at 23°C. Animals were fed and weighed 1 h before the onset of the dark cycle. All animals were given free access to water. Care of the rats was supervised by veterinarians and was in accordance with the policies of the Medical Center Laboratory Animal Care and Use Committee.

Ralston Purina Diets 5755M-S (Diet A, Basal Control Purina Diet) and 5755M-9 (Diet B, Vitamin-Fortified Purina Diet) were purchased from Purina Mills (Richmond, IN). The test diets were developed by Purina Mills according to the specifications of Yu et al. (1985). Diet B was developed (Yu et al. 1985) to ensure that the experimental animals consuming 60% of ad libitum intake of Diet A received the same absolute amounts of vitamins and choline chloride as the ad libitum diet animals (Purina Mills). The composition of the two diets is presented in **Table 1**. Animals were acclimated to the powdered diet for a minimum of 3 wk before calorimetry. Food consumption of each animal was measured daily and body weights were measured every 3 d.

Before the initial calorimetry and feeding restriction began, the rats were paired by weight and randomly assigned to the ad libitum or restricted groups. Each group of adult animals consisted of six animals with weights ranging from 338 to 408 g. After baseline calorimetry experiments and nuclear magnetic resonance (NMR) measurements of fat mass were conducted, the experimental feeding protocol was initiated. The ad libitum group was given Diet A in a large enough quantity to ensure that they would have food available for an entire 24-h period. The restricted animals were fed Diet B in an amount equivalent to 60% of the total amount of food consumed by the paired ad libitum diet animal during the previous 24-h period. For example, if the unrestricted animal consumed 15 g of food the paired restricted animal would receive 9 g of food on the following day. Each pair of animals was on the experimental feeding protocol for 6 wk. At completion of the experimental feeding period, the calorimetry measurements and NMR measurements of fat mass were repeated.

TABLE 1

Composition of diets[1]

Dietary component	Diet A	Diet B
	g/100 g diet	
Casein	21.00	21.00
Sucrose	15.00	15.00
Non-nutritive fiber (Solka Floc)	3.00	3.00
Corn oil	10.00	10.00
Dextrin	43.65	42.19
DL-Methionine	0.15	0.15
Vitamin mix[2]	2.00	3.33
Choline chloride	0.20	0.33
Mineral mix[3]	5.00	5.00

[1]Animals receiving Diet B at 60% restriction obtained the same absolute amounts of vitamin mix and choline chloride as unrestricted animals receiving Diet A. Both diets provided 17.45 kJ/g.

[2]Vitamin mix (Ralston Purina, Purina Mills, Richmond, IN) had the following composition per kg diet as fed in Diet A: 20 mg thiamin hydrochloride; 20 mg riboflavin; 90 mg niacin; 20 mg pyridoxine hydrochloride; 60 mg D-calcium pantothenate; 4 mg folic acid; 0.4 mg D-biotin; 200 mg I-inositol, 20 μg vitamin B-12; 20 mg menadione sodium bisulfite; 6600 μg RE retinyl acetate; 55 μg cholecalciferol; 50 mg all-*rac*-α-tocopherol acetate.

[3]Mineral mix (Ralston Purina No. 11) had the following composition per kg of diet as fed in Diet A: 7.5 g calcium; 4.5 g phosphorus; 4.6 g potassium; 2.9 g sodium; 2.9 g chloride; 0.65 g magnesium; 65 mg manganese; 60 mg iron; 20 mg zinc; 15 mg copper; 5 mg fluoride; 3.2 mg cobalt; 3 mg chromium; 0.6 mg iodine; 0.8 mg molybdenum; 0.2 mg selenium.

Total body water and fat contents were measured indirectly by NMR spectroscopy (Robinson et al. 1990). Rats were transported by vehicle in covered plastic carrying cages with raised stainless steel mesh floors from the Animal Resource Facility to the Center for Noninvasive Diagnosis (CNID) for NMR spectroscopy. The animals were provided with food and water during the time they were at the CNID. Nuclear magnetic resonance measurements were made on 4 rats/wk, and 3–7 d were allowed to elapse before the calorimetry protocol.

Anesthetized rats were placed individually in a 1.5 Tesla General Electric Medical Systems Signa Unit

(Milwaukee, WI). Using a pulse sequence consisting of a hard rectangular pulse with no slice selection, single excitation ^1H NMR spectra was obtained from each rat. Upon completion of the NMR measurements, rats were transported back to the Animal Resource Facility and were monitored through complete recovery from anesthesia.

The in vivo fat mass based on NMR was calculated according to the regression equation developed by Robinson et al. (1990). Lean body mass was calculated by subtracting in vivo fat mass determined by NMR from the total body weight.

A convective direct calorimeter designed and constructed by Alpert (1989) was used for direct measurement of the animals' metabolic rates. In this instrument, a current of air is circulated in the calorimeter. The animal is completely shielded from the air flow. Sensible heat loss from the animal is transferred to the recirculating air current by radiation and convection. The heat is extracted from the air and is measured by differential electronic techniques. The instrument is linear in the range of 0 to 8 W, has a minimum detectability of ~0.05 W, a temperature range of 0 to 40°C, and a response time of 2.3 min. Calibration was performed using a heater made of nichrome wire wound onto a lightweight composition form. System temperatures were measured by means of copper-constantan thermocouples. To date, a total of 44 calibration runs have been performed, yielding an average coefficient of variation of 0.010.

The animals' insensible heat loss rate was measured by using two Rototronic Hygroskop (Huntington Station, NY) humidity sensors (model HTS) and a General Vacuum (San Diego, CA) mass transport flow meter (Model FS6-41). The input air was filtered, dried and brought to the same temperature as the calorimeter. The relative humidity was then measured on both input and output, while the flow rate was measured on output. In calculating insensible heat loss, it was assumed that evaporation occurred at a body temperature of 37°C; the value of the latent heat of vaporization was taken to be 2.41 kJ/g. Flow rates were held close to 1.0 ± 0.02 L/min. The humidity sensors were calibrated at 35, 50 and 80% relative humidity using chemical ampules purchased from the sensor manufacturer. Flow rates were determined by use of Precision Scientific (Chicago, IL) wet test meter (model no. 63115). The error in the determination of the insensible heat loss rate is estimated to be ±5%, but because the insensible heat loss rate is <20% of the total heat expenditure in the temperature range used, the overall contribution to the heat loss rate error is about the same as the sensible heat loss rate, i.e., ±1%.

Calorimeter chambers were ~19 cm long, 14 cm wide and 14 cm high. The floor of the chamber was a stainless steel mesh on a pan of mineral oil to receive and cover collected urine and feces to prevent evaporation. The mesh floor was offset by 1 cm from the oil level, ensuring that the animals did not come in contact with the oil. Drinking water (20 mL) was freely available to the rat through an internal tube sealed with a displaceable stainless steel ball.

The calorimeter was turned on by 0800 h to allow for instrument stabilization. Frequently, the instrument ran continuously between experiments. At ~1200 h the rat was transported from the Animal Resource Facility to the Physics Building where the calorimeter was located, a distance of 183 m. The rat was transported a minimum of 3 h before being introduced into the calorimeter and was provided with free access to food and water throughout this period. During this time instrument baseline measurements were being recorded, which allowed the animal to become accustomed to the noise produced by the calorimeter and data recorder.

A single rat was placed in the calorimeter at 1500 h on d 1 and remained in the instrument until 1800 h on d 2 of the experiment. The 12 h light:dark cycle was maintained during the entire period the rat was in the calorimeter, and the rat was provided with water, but not food, throughout the experiment. Data collected during the first 3 h (1500–1800 h) were discarded to avoid transient effects of the animal's response to the change in its immediate environment and equipment shock when the chamber was opened. Data for the 24-h period (d 1, 1800 h to d 2, 1800 h) were retained for analysis. The rat was removed from the calorimeter at 1800 h and transported immediately to the Animal Resource Facility. The instrument continued to run until ~2300 h to obtain baseline data at steady state. At this point the calorimeter chamber was cleaned and prepared for the next animal.

Data were collected with a John Fluke (Mountlake Terrace, WA) data logger (model 2200B) at 15-min intervals; however, only the data collected at the hour and half-hour were used in the analysis. The average metabolic rate for the entire 24-h period was determined by averaging these 47 data points. The basal metabolic rate was determined by sorting the data collected during the entire 24-h period into an ascending order and averaging the five lowest points. The rationale for determining basal metabolic rate in this manner was that the lowest metabolic rate measurements should be representative of the animal's metabolic rate under basal conditions in the absence of food intake. Metabolic rate is expressed in watts.

Differences between mean values were analyzed by the paired Student's t test, with significance of the difference between mean values being assumed at $P \leq 0.05$ using a two-tailed test. Data were analyzed using a Litton Monroe (Orange, NJ) Statistical Programmable Printing Calculator (model 1860). Split-plot ANOVA was used to examine differences of the average and basal metabolic rate measurements obtained between groups and within groups during the

TABLE 2

Nuclear magnetic resonance measurements of fat mass and estimation of lean body mass of energy-restricted and unrestricted rats[1]

Group	Pre-energy restriction			Post-energy restriction		
	Body weight	Fat mass	LBM	Body weight	Fat mass	LBM
			g			
1	364.3 ± 8.4	80.6 ± 9.5	283.7 ± 9.3	395.3 ± 8.0	82.8 ± 11.4	312.4 ± 11.7
2	363.4 ± 6.5	80.1 ± 5.5	283.4 ± 8.7	287.5 ± 8.5	26.9 ± 7.8	260.5 ± 4.0
P value	NS	NS	NS	≤0.001	≤0.01	≤0.01

[1]Values are means ± SEM, n = 6. Group 1, rats not energy restricted; Group 2, energy-restricted rats. LBM = lean body mass; NS = no significant difference between mean values of Groups 1 and 2.

12-h light:dark cycle. Significance between groups was assumed at $P \leq 0.05$ and within groups at $P \leq 0.01$. Values reported are means ± SEM with the exception of Figure 2, where the values are means ± standard error of the estimate.

RESULTS

Calculations of body weight and body composition by NMR spectroscopy are presented in **Table 2**. Initial measurements of body weight, fat mass and fat free mass were not significantly different between randomized groups. Decreased body mass as a result of the 6-wk energy restriction of adult rats was characterized by a 66% decrease in fat mass measured by NMR spectroscopy ($P \leq 0.01$) and an 8% reduction in calculated lean body mass ($P \leq 0.01$).

The mean weight gain of rats allowed ad libitum access to food was 37.7 ± 4.4 g and the mean weight loss of energy-restricted rats was 58.6 ± 5.1 g over the 6-wk experimental period (**Fig. 1**). The difference in weight between groups was significant ($P \leq 0.05$).

In the restricted group, fat mass was 10% of body weight, whereas the ad libitum group had 21% of body weight as fat mass. The absolute body weight of ad libitum diet rats increased an average of 9% during the experimental period, whereas absolute fat mass and lean body mass increased by an average of 10 and 3%, respectively.

Results of measurements of average metabolic rate and basal metabolic rate by direct calorimetry are presented in **Table 3**. Data are presented per kilogram of body weight, per kilogram of lean body mass, and per kilogram of body weight to the 2/3 and 3/4 power. Normalization of data to kilogram of body weight resulted in no significant differences between the ad libitum diet rats (Group 1) and the energy-restricted rats (Group 2). When the data were normalized to lean body mass, however, metabolic rates of the restricted rats were significantly lower than those of the ad libitum diet rats. In addition, when the data were normalized to body weight to the 2/3 and 3/4 power, which are commonly used methods of expressing metabolic mass (Heusner 1985), metabolic rates of the restricted group were again significantly lower than those of the unrestricted group. **Figure 2** demonstrates basal metabolic rate vs. body weight for ad libitum diet Sprague-Dawley rats (n = 96). Plotted on this curve are the data for our control and experimental Fisher 344 rats. This figure emphasizes that the basal metabolic rate vs. body weight relationship for Fisher-344 rats allowed ad libitum access to food is virtually identical to that for Sprague-Dawley rats allowed ad libitum access to food, whereas restricted Fisher 344 rats have a much lower basal metabolic rate than ad libitum diet Sprague-Dawley rats of similar weight.

Average metabolic rate measurements made during the 12-h light period were significantly lower than those observed during the dark period ($P \leq 0.01$), as expected for a nocturnal animal. These effects were demonstrated in both the energy-restricted and unrestricted groups. A significant difference ($P \leq 0.05$) for

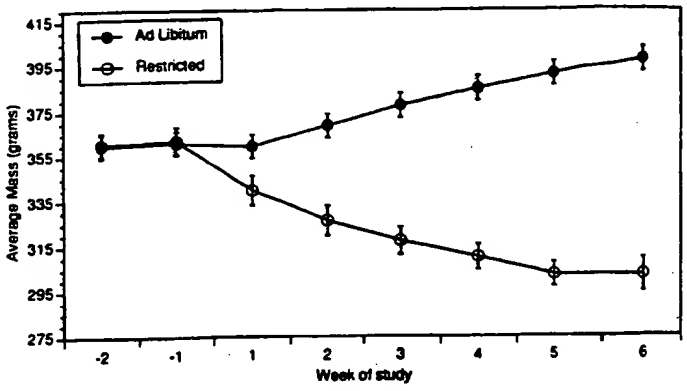


FIGURE 1 Average body mass for each group at baseline and at the end of each week during the experimental feeding phase. Rats allowed ad libitum access to food, •; rats fed energy-restricted diet, o. Points are means ± SEM; n = 6.

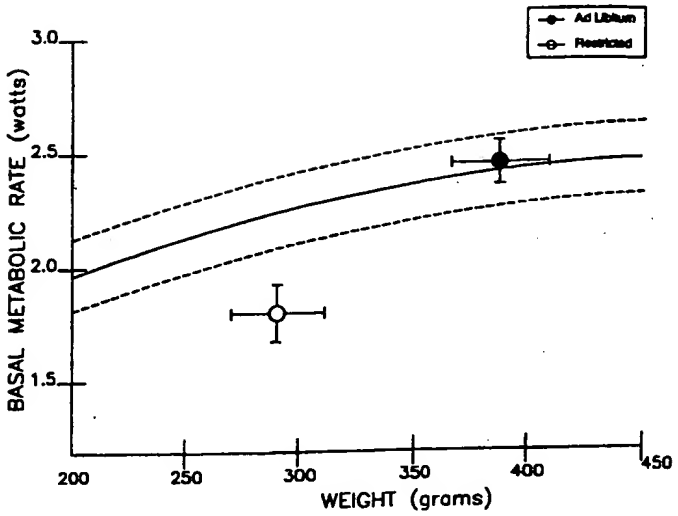


FIGURE 2 Least squares fit curve of average basal metabolic rate vs. total body weight for unfed normal male Sprague-Dawley rats; $n = 96$ (S. Alpert unpublished data). The solid line represents the mean and the dashed lines represent the standard error of the estimate (± 0.13 W). Fisher 344 rats allowed ad libitum access to food, •; Fisher 344 rats fed energy-restricted diet, o. Points are means ± standard error of the estimate; $n = 6$.

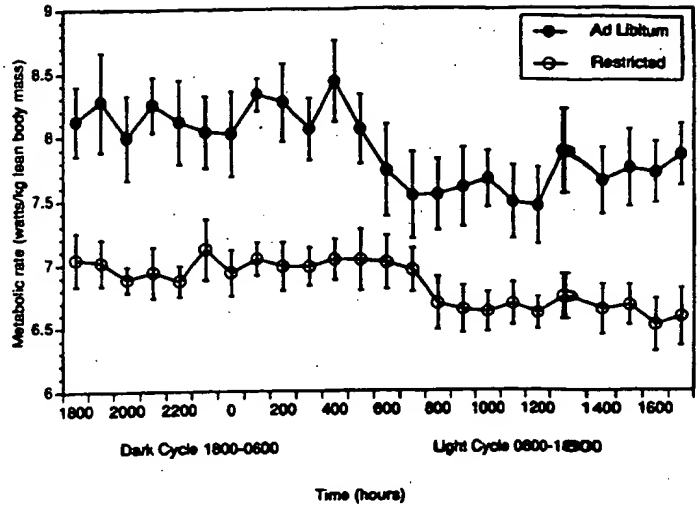


FIGURE 3 Average metabolic rate over the 24 h direct calorimetry period. Metabolic rate is normalized to kilograms of lean body mass. Rats allowed ad libitum access to food, •; rats fed energy-restricted diet, o. Points are means ± SEM; $n = 6$.

average metabolic rate between the ad libitum and energy-restricted groups was obtained during the light and dark cycles. Basal metabolic rate measured during the light cycle was significantly lower for the energy-restricted group ($P \leq 0.05$). Interaction between the feeding protocol and the 12-h light:dark cycle as determined by ANOVA was not significant ($P \geq 0.05$). Diurnal variation of average metabolic rate is illustrated in **Figure 3**.

Baseline measurements of the average daily energy intake for the energy-restricted and unrestricted animals were similar (0.63 ± 0.01 kJ/g·d). During wk 1 of restriction, the absolute energy intake of the restricted group was reduced by 40% from baseline (0.38 ± 0.02 kJ/g·d). Energy intake relative to body weight was reduced from baseline by an average of 27% during wk 2–5 and 21% during wk 6 of the experimental restriction period (0.46 ± 0.02 kJ/g·d). The average energy intake per gram of body weight was constant for the ad libitum group.

DISCUSSION

The average and basal metabolic rates obtained by direct calorimetry in our experiments demonstrated that the energy-restricted animals had lower metabolic rates than the ad libitum diet animals (Table 3). These results were obtained when data were normalized to kilogram of lean body mass and to body weight to the 2/3 and 3/4 power. It is noteworthy that these methods used to normalize data relative to metabolic mass demonstrated significant differences between ad libitum and energy-restricted groups.

The restricted group had a relatively larger proportion of lean body mass to body weight, yet the

TABLE 3

***Average and basal metabolic rate as measured by direct calorimetry of energy-restricted and unrestricted rats*[1,2]**

Group	Average metabolic rate				Basal metabolic rate			
	BW	LBM	BW	BW	BW	LBM	BW	BW
	W/kg		*W/kg*$^{2/3}$	*W/kg*$^{3/4}$	*W/kg*		*W/kg*$^{2/3}$	*W/kg*$^{3/4}$
1	6.35 ± 0.09	7.92 ± 0.31	4.63 ± 0.05	5.01 ± 0.06	5.70 ± 0.12	7.11 ± 0.31	4.15 ± 0.07	4.50 ± 0.08
2	6.10 ± 0.09	6.85 ± 0.20	4.05 ± 0.07	4.49 ± 0.07	5.55 ± 0.06	6.23 ± 0.19	3.68 ± 0.05	4.08 ± 0.05
P value	NS	≤0.01	≤0.001	≤0.01	NS	≤0.01	≤0.01	≤0.01

[1]Values are means ± SEM, $n = 6$. Group 1, rats not energy-restricted; Group 2, rats energy-restricted.
[2]Abbreviations used: BW, body weight, LBM, lean body mass; NS, no significant difference between mean values of Groups 1 and 2.

average metabolic rate per kilogram of lean body mass was significantly lower than for the ad libitum group. The 21% body fat in ad libitum diet rats in our study is similar to that reported by McCarter and McGee (1989) and Beauchene et al. (1986). Previously reported measurements of body fat in restricted rats have ranged from 10% (Masoro et al. 1982, McCarter et al. 1985) to 15% (Beauchene et al. 1986, McCarter and McGee 1989). The 10% body fat in energy-restricted rats in our study is similar to that reported by McCarter et al. (1985) and Masoro et al. (1982).

The metabolic rates normalized to kilogram of lean body mass are in apparent disagreement with those reported by McCarter et al. (1985), who found that chronic energy restriction did not affect metabolic rate. In addition, McCarter and McGee (1989) reported that reduced metabolic rate was a transient effect of energy restriction. Our findings, obtained under different experimental conditions, suggest that decreases in metabolic rate in adult animals produced by chronic energy restriction are not transient. Hill et al. (1988) reported that after 94–128 d of energy restriction at 80% of ad libitum consumption, the absolute energy expenditure of the energy-restricted rats was significantly lower than for the ad libitum diet rats. This difference was lost when the data were expressed per kilogram of body weight; however, the data were not expressed per kilogram of lean body mass, which may have shown a difference between groups because the energy-restricted rats had a significantly lower absolute fat-free dry weight than the ad libitum diet rats. Ballor (1991) reported that after 9 wk of moderately severe and severe dietary restriction, 23-h relative resting metabolic rate was significantly depressed compared with that of ad libitum diet, cage-confined female rats. These findings are in agreement with the results of the present study and support the suggestion that reduction of metabolic rate is not a transient effect of energy restriction. Conditions under which the metabolic rate measurements were collected are similar to those of the present study.

In work on Sprague-Dawley rats, Alpert has compiled a curve of basal metabolic rate vs. total body weight (Fig. 2) for 96 unfed normal animals (Alpert, S. S. unpublished data). Our averaged results for the control and experimental Fisher-344 rats are plotted on the same graph. It is clear that the control group is consistent with the results for Sprague-Dawley rats, whereas the basal metabolic rate for the energy-restricted group falls below the basal metabolic rate for ad libitum diet animals of equivalent weight, supporting the data shown in Table 3.

A possible explanation for discrepancies between our experiments and those previously reported (McCarter and McGee 1989, McCarter et al. 1985), is the differing ages of the animals studied. Mohan and Narasinga Rao (1983) concluded that energy restriction in growing animals does not result in decreased basal metabolism because decreased body size is a result of growth retardation. Chronic energy restriction of adult rats results in decreased basal metabolism due to changes in body composition and the loss of metabolizing tissue. Adaptation of the adult animal to energy restriction may involve conservative mechanisms to minimize energy losses as heat and the available energy may be more efficiently used for ATP synthesis for biosynthetic and tissue repair processes, i.e., there are increases in metabolic efficiency (Weindruch and Walford 1988). Increased metabolic efficiency would be observed in restricted animals as a tighter coupling of mitochondrial oxidative phosphorylation (Weindruch and Walford 1988), similar to that found in physical conditioning (Holloszy and Booth 1976) and would be reflected as increased ATP production per unit of heat production, relative to ad libitum diet animals, for a standard energy intake and level of oxygen utilization. Weight would decrease in restricted animals until the increases in metabolic efficiency that could be achieved by the individual animal produced adequate ATP for tissue maintenance and physical activity. Physical activity may be required to maintain core body temperature in the face of reduced heat generation occurring relative to ad libitum diet animals (Duffy et al. 1989). In the absence of activity, restricted animals may enter a torporous state (Duffy et al. 1989). Several studies have demonstrated reductions in heat production, basal metabolic rate and the thermic effect of food in restricted animals (Duffy et al. 1989, Forsum et al. 1981, Hill et al. 1985), providing evidence of increased ability to convert ingested energy to ATP. Bertrand et al. (1980) found that decreased fat mass is not a factor in the life-prolonging effect of energy restriction, but rather that there is a positive correlation between fat mass and length of life for the restricted animals. This suggests that animals that can increase metabolic efficiency to the greatest level, thus sparing fat, are those that live longest. This mechanism seems to be beneficial for longevity, but may be at least transiently detrimental when returning to ad libitum feeding because the animal has an increased ability to store energy (Hill et al. 1984).

Energy restriction may also induce gene expression of enzymes that protect against cellular damage by free radicals and other reactive intermediates, e.g., glutathione. Energy restriction has been shown to result in both increases and decreases in genetic expression, in each case mimicking genetic expression in non-food-restricted animals of younger chronological age (Cheung and Richardson 1982, Richardson et al. 1987). Energy intake, acting through glucose, insulin and/or other endocrine factors, may be a regulator of genetic expression related to aging and disease susceptibility (Masoro 1988, 1989 and 1990). It is

possible that improved control of plasma glucose and insulin concentrations may be linked to increased energy efficiency of energy-restricted rats (Weindruch and Walford 1988).

Comparison of metabolic rates during the 12-h light and dark periods demonstrated that the restricted rats exhibited a normal diurnal variation in metabolic rate (Fig. 3). The restricted animals did not exhibit as much fluctuation within the 12-h light: dark cycles as the unrestricted animals. This suggests that energy restriction may induce more precise control of all metabolic functions. These findings are in contrast to those reported by McCarter and McGee (1989), who demonstrated that after 4 wk of energy restriction, the diurnal patterns between groups were very similar with the exception of a 2-h period 11–14 h after the restricted animals had been fed, when the unrestricted group had a higher metabolic rate than the restricted group. The conditions of our study differ from those of McCarter and McGee (1989) in that both restricted and unrestricted groups were deprived of food during the entire 24-h experiment.

The average intake of the ad libitum diet rats was similar to previously reported data on 6 to 7-mo-old Fisher 344 rats allowed ad libitum access to food (Masoro et al. 1982). Energy-restricted rats consumed fewer kilojoules per gram of body weight than the ad libitum group throughout the 6-wk restricted feeding experiment. During the period of energy restriction, there was a rapid adjustment of body weight to sustain intake at a level of 0.46 kJ/g body wt. These findings raise the question of whether this is the minimum amount of energy per gram of body weight required to maintain the animals in a healthy state. These results differ from data reported by Masoro et al. (1982) for Fisher-344 rats (6–9 mo old) in which the rate of kilojoules consumed per gram of body weight was the same for rats receiving an unrestricted or restricted diet (0.59–0.63 kJ/(g·d). However, the animals in the Masoro et al. (1982) study were restricted at 6 wk of age. We suggest that these conflicting results are due to different age-associated metabolic adaptation to reduced energy intake.

Conditions of this study included the removal of experimental animals from their normal environment for NMR and calorimetry measurements and depriving the animals of food during the 24 h they were in the calorimeter. After NMR spectroscopy for indirect measurement of fat mass and estimation of lean body mass the animals needed a few days to completely recover from the effects of anesthesia. During this period the animals experienced some weight loss, and the ad libitum group temporarily reduced their food consumption. However, NMR spectroscopy proved to be a convenient and suitable method for measuring fat stores, and the reduction of food consumption was transient. The coefficient of variation of basal metabolic rate per gram of lean body mass was small (3.9 ± 2.7%). Basal metabolic rate and average metabolic rate were readily apparent from the 24-h tracings, and values were obtained without the transient effects of energy intake. In addition, our experimental results demonstrate that restricted animals maintain reductions in basal metabolic rate and average metabolic rate throughout the period of acute energy deprivation.

The results of these experiments support the hypothesis that energy restriction of adult rats results in decreases in both average metabolic rate and basal metabolic rate compared with ad libitum diet rats. This does not rule out the possibility that energy restriction beyond 6 wk will cause the difference in metabolic rate of adult rats to be lost; however we found no indications at the end of the experiment suggesting this will occur. When the heat loss data were expressed relative to kilogram of lean body mass, a 14% decrease in average metabolic rate was obtained and basal metabolism was decreased by 12%. These results do not support that conclusion drawn by McCarter and McGee (1989) that decreased metabolic rate is a transient effect of energy restriction.

It is likely that the responses to energy restriction result from a number of biological and/or physiological alterations, including changes in metabolic rate. The findings of this study indicate that further comparison of the effects of chronic energy restriction on the metabolism of adult and weanling rats and the association between energy restriction and increased life span is warranted.

ACKNOWLEDGMENT

We would like to thank the personnel at the Center for Non-Invasive Diagnosis, University of New Mexico, for conducting the in vivo NMR measurements.

LITERATURE CITED

Alpert, S. S. (March 29, 1989) A convective direct calorimeter for small animals, neonates, and infants. University of New Mexico Patent disclosure no. 262. U.S. Patent No. 5,135,311 (August 4, 1992).

Ballor, D. L. (1991) Effect of dietary restriction and/or exercise on 23-h metabolic rate and body composition in female rats. J. Appl. Physiol. 71: 801–806.

Beauchene, R. E., Bales, C. W., Bragg, C. S., Hawkins, S. T. & Mason, R. L. (1986) Effect of age of initiation of feed restriction on growth, body composition, and longevity of rats. J. Gerontol. 41: 13–19.

Bertrand, H. A., Lynd, F. T., Masoro, E. J. & Yu, B. P. (1980) Changes in adipose mass and cellularity through the adult life of rats fed ad libitum or a life-prolonging restricted diet. J. Gerontol. 35: 827–835.

Cheung, H. T. & Richardson, A. (1982) The relationship between age-related changes in gene expression, protein turnover and the

responsiveness of an organism to stimuli. Life Sci. 31: 605–613.
Duffy, P. H., Feuers, R. J., Leakey, J. A., Nakamura, K. D., Turturro, A. & Hart, R. W. (1989) Effects of chronic caloric restriction on physiological variables related to energy metabolism in the male Fischer 344 rat. Mech. Ageing and Dev. 48: 117–133.
Forsum, E., Hillman, P. E. & Nesheim, M. C. (1981) Effect of energy restriction on total heat production, basal metabolic rate, and specific dynamic action of food in rats. J. Nutr. 111: 1691–1697.
Harman, D. (1981) The aging process. Proc. Natl. Acad. Sci. U.S.A. 78: 7124–7128.
Heusner, A. A. (1985) Body size and energy metabolism. Ann. Rev. Nutr. 5: 267–293.
Hill, J. O., Fried, S. K. & DiGirolamo, M. (1984) Effects of fasting and restricted refeeding on utilization of ingested energy in rats. Am. J. Physiol. 247: R318–R327.
Hill, J. O., Latiff, A. & DiGirolamo, M. (1985) Effects of variable caloric restriction on utilization of ingested energy in rats. Am. J. Physiol. 248: R549–R559.
Hill, J. O., Anderson, J. C., Lin, D. & Yakubu, F. (1988) Effects of meal frequency on energy utilization in rats. Am. J. Physiol. 255: R616–R621.
Holloszy, J. O. & Booth, F. W. (1976) Biochemical adaptations to endurance exercise in muscle. Annu. Rev. Physiol. 38: 273–291.
Masoro, E. J., Yu, B. P. & Bertrand, H. A. (1982) Action of food restriction in delaying the aging process. Proc. Natl. Acad. Sci. U.S.A. 79: 4239–4241.
Masoro, E. J. (1984) Nutrition as a modulator of the aging process. Physiologist 27: 98–101.
Masoro, E. J. (1988) MINIREVIEW: Food restriction in rodents: an evaluation of its role in the study of aging. J. Gerontol. Biol. Sci. 43: B59–B64.
Masoro, E. J. (1989) Nutrition and aging in animal models. In: Nutrition, Aging, and the Elderly (Munro, H. N. & D. E. Danford, eds.), pp. 25–42. Plenum Press, New York, NY.
Masoro, E. J. (1990) Assessment of nutritional components in prolongation of life and health by diet (42985). Proc. Soc. Exp. Biol. Med. 193: 31–34.
McCarter, R., Masoro, E. J. & Yu, B. P. (1985) Does food restriction retard aging by reducing the metabolic rate? Am. J. Physiol. 248: E488–E490.
McCarter, R. J. & McGee, J. R. (1989) Transient reduction of metabolic rate by food restriction. Am. J. Physiol. 257: E175–E179.
Mohan, P. F. & Narasinga Rao, B. S. (1983) Adaptation to underfeeding in growing rats. Effect of energy restriction at two dietary protein levels on growth, feed efficiency, basal metabolism and body composition. J. Nutr. 113: 79–85.
Richardson, A., Butler, J. A., Rutherford, M. S., Sensei, L., Gu, M., Fernadez, G. & Chiang, W. (1987) Effect of age and dietary restriction on the expression of alpha 2U-globulin. J. Biol. Chem. 262: 12821–12825.
Robinson, J. W., Masoro, E. J., Sabatino, F., Fullerton, G. D. & Williams, R. F. (1990) Absolute measurement of extractable fat in whole rats by NMR. Mag. Reson. Med. 2: 1096.
Sacher, G. A. (1977) Life table modifications and life prolongation. In: Handbook of the Biology of Aging (Finch, C. E. & L. Hayflick, eds.), pp. 582–638. Van Nostrand Reinhold, New York, NY.
Sacher, G. A. & Duffy, P. H. (1979) Genetic relation of life span to metabolic rate for inbred mouse strains and their hybrids. Fed. Proc. 38: 184–188.
Weindruch, R. & Walford, R. L. (1988) The Retardation of Aging and Disease by Dietary Restriction. Charles C Thomas Publishing, Springfield, IL.
Yu, B. P., Masoro, E. J., Murata, I., Bertrand, H. A. & Lynd, F. T. (1982) Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: longevity, growth, lean body mass and disease. J. Gerontol. 37: 130–141.
Yu, B. P., Masoro, E. J. & McMahan, C. A. (1985) Nutritional influences on aging of Fischer 344 rats. I. Physical, metabolic, and longevity characteristics. J. Gerontol. 40: 657–670.

MINIREVIEW

Nuclear Transplantation as a Method for Cloning Embryos (43112)

RANDALL S. PRATHER
Department of Animal Sciences, University of Missouri, Columbia, Missouri 65211

Historical Perspective

The concept of transferring nuclei from multicell stage embryos to enucleated oocytes was first proposed by Spemann in 1938 (1). Spemann was a developmental biologist whose concern at the time was that of nuclear equivalence. The idea of the basic composition of the nuclei changing during early embryo development gave rise to the theory that nuclei from different tissues were not equal. To answer this question, Spemann proposed an experiment to transfer nuclei from progressively more advanced embryos to enucleated, activated oocytes. The conclusion of the experiment would be determined when inequivalence was achieved and the transferred nucleus would not support development to a mature adult. Results from Spemann's experiment were first reported in 1952 by Briggs and King (2) in the amphibian *Rana pipiens*. They (2) showed that nuclei from preblastula stage embryos could promote development to the blastula stage. In addition, they alluded to the observation that nuclei from beyond the blastula stage had a lesser probability of promoting development after nuclear transfer.

The spin-off of such a project is the possibility of cloning. Since all of the nuclei of the early embryo are presumed to be identical (all containing the same complement of genetic material), subsequent transfer to enucleated, activated oocytes with development to term should result in genetically identical individuals (the reality of identical individuals will be discussed later). This procedure, in combination with serial nuclear transfer (i.e., growing the first nuclear transfer embryos to the donor cell stage and then repeating the procedures) could, theoretically, result in an unlimited number of identical individuals. The possibility of this in mammals became much more likely after the successes of such procedures were reported in 1986 by Willadsen (3) and Prather *et al.* (4) in sheep and cattle, respectively.

In this review I hope to provide information on the procedures for nuclear transfer, present the concept of nuclear remodeling and reprogramming, list the factors to consider regarding the similarity of nuclear transfer embryos, and what will be necessary for commercialization of such technology.

0037-9727/1951-000752.00/0

Nuclear Transfer Procedures

The procedures for nuclear transfer are basically the same as those described by Briggs and King (2) for amphibians and adapted to mammals by McGrath and Solter (5). First, a group of recipient unfertilized oocytes is enucleated. This is accomplished after treating the oocytes with microfilament- and microtubule-inhibiting drugs such as cytochalasin B and colchicine. With the disruption of the cytoskeleton, the plasma membrane is much less likely to rupture. The recipient oocyte is held in place by aspiration with a micropipette (Fig. 1A) and another micropipette is punctured through the zona pellucida. The polar body and metaphase chromosomes are aspirated into the pipette. When the pipette is removed, the plasma membrane pinches off, forming two membrane-enclosed vesicles (one inside the pipette and one inside the zona pellucida). Originally, the aspiration was done blind, but visualization under ultraviolet light with a DNA stain permits successful enucleation every time (6). Next, the donor embryo is aspirated against the holding pipette and a single blastomere, or karyoplast, is aspirated into the transfer pipette (Fig. 1B). The transfer pipette is then inserted into the perivitelline space of the enucleated oocyte and the karyoplast is deposited (Fig. 1C). The two cells (donor karyoplast and recipient oocyte) are then allowed to regain their spherical shape (Fig. 1D). The resulting nuclear transfer embryo is placed

between two electrodes and an electrical pulse that causes a transient breakdown of the plasma membranes is applied. When the membranes reform, small channels are created that, due to their thermodynamic instability, enlarge forming a single cell (7, 8). The electrical pulse required for cell fusion also results in the activation of the oocyte, i.e., simulation of fertilization, thus setting into motion the events necessary for early development.

Nuclear Remodeling

After the successful transfer of a karyoplast to an enucleated, activated oocyte, the transferred nucleus undergoes remodeling such that it morphologically and metabolically resembles a pronucleus. In amphibians, this is observed by an increase in diameter of the transferred nucleus (9) and the disappearance of nucleoli within the transferred nucleus (10). In mammals, where the nucleoli only undergo ultrastructural modifications during early development and not the complete disappearance as in amphibians, the nucleoli revert to their early cleavage stage morphology (11). In addition, there is significant swelling of the transferred nuclei in mice (11, 12), rabbits (13), and pigs (14). There appears to be a 1½-hr window around the time of activation of the oocyte in which chromatin can be remodeled to be similar to pronuclei (11, 12). If the nuclei are transferred outside this window, then the nuclei either condense or fail to swell. In the rabbit, the timing criteria are even more confining since optimum activation rates are achieved only during a narrow window after ovulation (15). Interestingly, when zygotes, which are outside this window, are used as recipients development does not continue to term in cattle (16) or mice (17).

Theoretically, for optimum reprogramming, the nuclei should swell to a size similar to an endogenous pronucleus. Nuclear swelling is a result of the exchange of proteins between the cytoplasm and the nucleoplasm (18, 19). This exchange of proteins is thought to be the inducer of the swelling, not a consequence of nuclear swelling (20). Since there are components in the cytoplasm that affect nuclear volume, it is important to note that the early mammalian nuclear transfer experiments used recipient oocytes from which half of the cytoplasm was removed. This likely removed half of the components within the cytoplasm that are responsible for nuclear volume and subsequent reprogramming. A 2 × 2 factorial experiment was designed to evaluate the affect of cytoplasmic volume on nuclear swelling. Nuclear size was measured in embryos resulting from the transfer of an intact or half blastomere to either an intact or half oocyte. Neither the removal of half of the cytoplasm from the recipient oocyte, nor the transfer of half of a blastomere decreased the degree of swelling observed of the transferred nucleus (14). However, it is not yet known if these parameters are related to subsequent development.

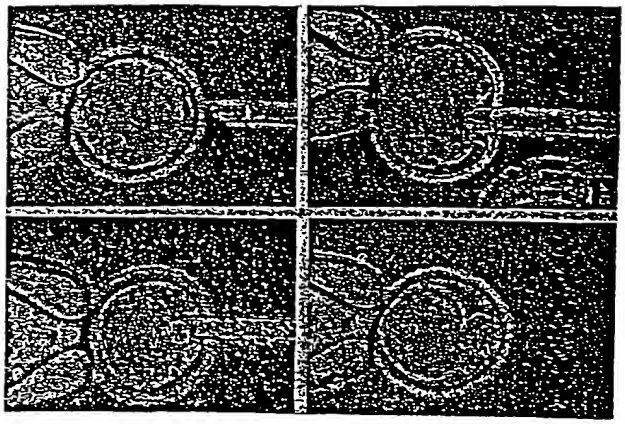


Figure 1. Nuclear transfer in cattle embryos. (A) Mature unfertilized oocyte (first polar body, a result of the completion of meiotic metaphase I, is denoted by the arrow). (B) Aspiration of blastomere containing donor nucleus. (C) Transfer of blastomere to enucleated oocyte. Arrow indicates the donor nucleus within the transfer pipette. (D) After transfer of donor blastomere, arrow indicates donor blastomere (original magnification ×400; diameter of the zona pellucida is about 150 μm). Reprinted with permission from Prather *et al.* (40).

In amphibians, after nuclear transfer, there is a shift of protein from the oocyte cytoplasm into the transferred nucleus and a shift of protein from the nucleus into the cytoplasm. This selective exchange of proteins between the nucleus and cytoplasm does not appear to be limited by the nuclear envelope, but by selective binding sites within the nucleus (21). In *R. pipiens*, nonhistone [^{3}H]tryptophan-containing proteins leave transferred endodermal nuclei, but [^{3}H]-lysine-containing proteins remain in the nucleus (18, 19). Synchronous with the release of labeled proteins is the acquisition of both acid and basic proteins by the nucleus (20).

In mammals, there is at least one suggestion of a similar exchange of nuclear lamins between transferred nuclei and cytoplasm. Nuclear lamins are a class of intermediate filaments that line the inner nuclear envelope and polymerize and depolymerize with the cell cycle. In the mouse, cow, and pig the A/C nuclear lamin epitope (antibody J9) becomes undetectable after the transition to zygotic control of development. In both the mouse and pig, if nuclei beyond the transition to zygotic control of development are transferred to enucleated activated oocytes, then the transferred nucleus acquires the lamin A/C epitope (Fig. 2) (22, 23). This is suggestive of acquisition of the lamin A/C from the cytoplasm of the oocyte. However, in the mouse, if nuclei are transferred to either an intact or enucleated zygote, then the transferred nuclei do not acquire the epitope (23). The inability of nuclear lamins to exchange between interphase cells has also been demonstrated in tissue culture (24). I hypothesize that the

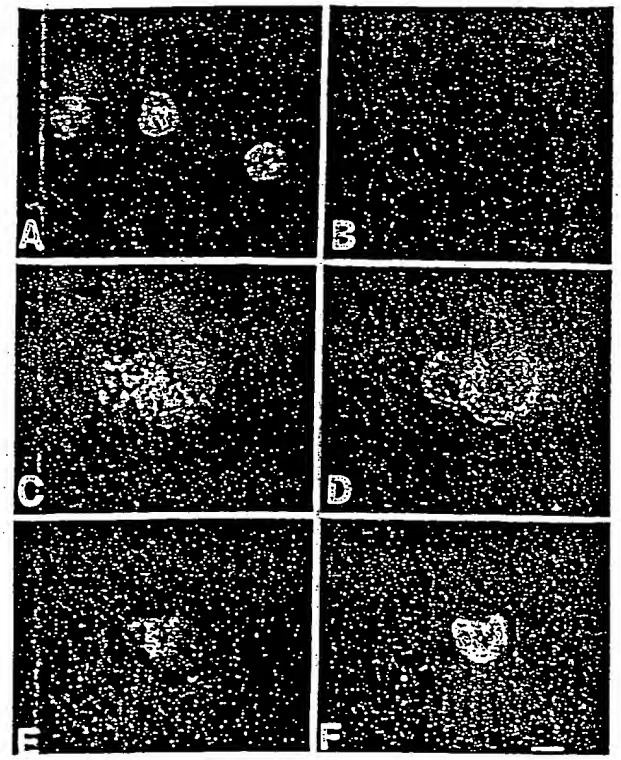


Figure 2. Nuclear lamin epitope after transfer of a 16-cell stage porcine nucleus to an activated, enucleated meiotic metaphase II arrested oocyte. DNA (Hoechst 33258) in cells from a 16-cell stage embryo (A), pronuclear stage egg (B), and activated egg after nuclear transfer (nucleus from 16-cell embryo in A; E), and corresponding lamin A/C (antibody J9) reactivity (B, D, and F, respectively). Note the absence of lamin A/C reactivity in the 16-cell stage blastomeres, whereas after transfer to an activated enucleated meiotic metaphase II oocyte and 2 hr of culture, the nucleus acquires the antigen. B, D, and E were photographed and developed under identical conditions. Cells in A, B, E, and F were mounted and stained on the same coverslip (bar = 10 μm). Reprinted with permission from Prather *et al.* (22).

inability to acquire the lamin A/C epitope is a result of the sequestering of cytoplasmic lamin A/C by the endogenous pronuclei. Thus, when an interphase cell is used as a recipient, the nuclei do not have access to the A/C lamin proteins within the cell. However, when a metaphase cell is the recipient, then the nuclear lamin proteins are dispersed in the cytoplasm and available to be incorporated into the nuclear envelope of the transferred nucleus.

Additional remodeling that is necessary before development can continue is a synchronization of the DNA synthetic phase of the donor and recipient cells. Nuclei that are in G_1 when transferred to an activated, enucleated oocyte need to complete DNA synthesis and progress to G_2 before the recipient cell attempts to divide. It is also important that nuclei in G_2 do not undergo DNA synthesis before mitosis, as the resulting embryo would be polyploid. The inability to complete DNA synthesis may be one of the most important factors affecting the resulting development. Most of the chromosomal abnormalities in amphibian nuclear transfer embryos can be traced to events that occur in the first cell cycle (25). The major problem in achieving synchrony between the donor and recipient cells is the fact that as a tissue differentiates the length of the cell cycle increases. These nuclei with long cell cycles, when transferred to an activated, enucleated oocyte, are required to complete DNA synthesis and prepare for another mitosis within an hour. Thus, relatively more differentiated nuclei are more likely to result in chromosomal abnormalities (26, 27), but any conclusions about differentiated cells versus less differentiated cells is confounded with the length of the cell cycle of the donor embryo.

Nuclear Reprogramming

The only indication, published to date, of reprogramming in mammals is the reprogramming of morphologic events. For example, if a cell from a 32-cell stage bovine embryo is transferred to an enucleated activated metaphase II oocyte and is not reprogrammed, the resulting embryo should attempt to form a blastocoele after one cleavage (at the 2-cell stage). If successful this would result in a trophoblastic vesicle that contains no inner cell mass, and hence no fetus. In addition, the absence of reprogramming would require that the nuclear transfer embryo be transferred to a surrogate mother than was synchronous with the donor 32-cell stage embryo. This would be true since domestic animals require close synchrony between the stage of the estrus cycle and stage of the developing embryo. If, instead, the 32-cell stage nucleus was reprogrammed to behave as a zygote, then the resulting embryo would begin compaction at the 32-cell stage, form a blastocoele at the 64-cell stage, and be transferred to a surrogate that ovulated synchronously with the nuclear transfer. Thus, a reprogrammed nucleus would retraverse the early cleavage stages followed by compaction and blastocoele formation, whereas control nonmanipulated embryos would attempt to continue their developmental sequence of events.

For a full appreciation of the biochemical reprogramming that presumably occurs after nuclear transfer in mammals, it should be understood that the early mammalian embryo does not begin producing RNA until a species-specific cell stage. Transcription can first be detected in the mouse embryo at the 2–cell stage (28), at the 4-cell stage in the pig (29) and rat (30), and at the 8- to 16-cell stage in the cow, rabbit, and sheep (31–33). Prior to this time the embryo relied upon RNA stored in the oocyte during oogenesis. Thus, for detectable biochemical reprogramming, nuclei need to be transferred at or after the transition to zygotic control of development. The most advanced nucleus to result in development to term by species is listed in Table I.

Table I. Relative Degree of Differentiation and Development after Nuclear Transfer to an Enucleated Activated Oocyte

Species	Stage of major transition to zygotic of development	Most advanced cell reported to result in term development
Pig (37)	4-Cell	4-Cell
Rabbit (13)	8- to 16-Cell	8-Cell
Cow (38–40)	8-Cell	32-Cell
Sheep (34)	8- to 16-Cell	Inner cell mass

The listing in Table I should not be interpreted to be maximum stage of development that will result in complete reprogramming, as few studies have attempted determine this parameter. The sheep should result in the most dramatic biochemical reprogramming since an inner cell-mass stage nucleus has resulted in term development (34).

In amphibians, the reprogramming is much more specifically described. Not only is there obvious morphologic reprogramming, similar to that described above, but there is also very specific biochemical reprogramming that occurs. Two of the most specific examples include the muscle-specific actin gene and the *5S*^{oocyte} gene. Muscle-specific actin is produced by new transcription and translation in the differentiating myotome cells of the gastrula stage embryo. After the transfer of postgastrula stage myotome cell nuclei to enucleated activated oocytes, the production of muscle-specific actin RNA ceases. Muscle-specific actin RNA synthesis is not reinitiated until the embryo develops to the gastrula stage, and then only in the differentiating myotome cells (35). Similarly, the *5S*^{oocyte} gene is translated and transcribed for a short period of time at the late blastula stage. Nuclei from beyond the blastula stage that are transferred to an enucleated activated oocyte transcribe the *5S*^{oocyte} gene for a short period as the resulting embryo passes through the blastula stage (36). Thus, the biochemical reprogramming that occurs in amphibians is very precise.

Identical Individuals

Genetic Variation. Since all of the nuclei of early embryos are presumed to be identical, the offspring resulting from nuclear transfer should all have identical nuclear genetics. However, if prior to nuclear transfer some chromosomal rearrangement occurs in a single blastomere of the donor embryo, then this genetic defect would be passed to all subsequent embryos. Some possibilities of chromosomal rearrangement include DNA rearrangements (as happens in the normal differentiation of immunoglobulins) (41), gene amplification, translocations, and diminution.

A second factor to consider is the non-nuclear genetics. Cytoplasmic inheritance would be important when the source of the recipient oocytes are not known, such as when using *in vitro* matured oocytes derived from slaughterhouse animals. The main source of cytoplasmic inheritance is likely from the mitochondria. Even if the recipient eggs are derived from the same breed of livestock, it is disturbing that within a single maternal line of cattle the mitochondrial genome has been observed to change (42). Since the nucleus directs the synthesis of proteins used in the mitochondria (43), the interaction between the two may be very important.

There may be other organelles within the cytoplasm that have their own genome. One example may be the centriole. Recent evidence suggests that basal bodies have their own genome (44). Basal bodies are derived from the centriole, and thus the conclusion that centrioles may have their own DNA. It is very interesting to note that centrioles are absent in mammals during the first few cleavages after fertilization (45, 46). Since the presence or absence of the centrioles is related to the shape of the mitotic spindle (46), it is interesting to speculate what would happen to the centrioles and shape of the mitotic spindle after the transfer of an embryonic cell which had centrioles to an oocyte which has no centrioles!

Whether differences in cytoplasmic inheritance as described above are important in determining subsequent development remains to be determined. The possibility of such factors affecting development does exist and should be investigated.

Phenotypic Variation. The factors that affect phenotypic variation include environment and genetics. Since the possible genetic variation has already been discussed, this section will focus on environmental affects on development.

Phenotypic variation exists even in monozygotic twins. Since both cytoplasmic and nuclear genetics are presumed to be identical in monozygotic twins, all variation observed is due to environmental factors. Interestingly, cattle embryos split at the morula stage result in calves that are considered monozygotic twins, but they do not always look alike. Differential migration of the melanocytes results in twins that have the same basic color pattern, but are not identical. For example, one of the twins may have a red patch over its eye, while the other has a similar patch below its eye (47). Embryos that result from nuclear transfer are in different environments from the moment of transfer. Thus, the differences in epigenetic phenomena, *in vitro* environment, uterine environment, neonatal environment, and postnatal environment all have effects upon the resulting phenotype (48).

The first requirement for nuclear transplant embryos to be identical is identical genetics, and the second requirement is identical environments. It is likely that identical genetics (both cytoplasmic and nuclear) can

be evaluated and controlled; however, an identical environment is much more difficult to control.

Lest one is left with the impression that nuclear transfer embryos, or for that matter twins, are not phenotypically similar, it is necessary to end this with additional information. Above the impression has been given that nuclear transfer embryos or twins are not phenotypically identical. Using a strict definition this is true; however, for most researchers an increase in genetic uniformity would be very advantageous for reducing the number of animals needed for statistically valid results. Genetically similar animals not only have more similar growth characteristics than unrelated controls, but also have more similar behavior patterns (reviewed by Biggers (49)). Thus, from a research standpoint genetically uniform animals would be very useful. There would also be advantages for commercial livestock production as described below.

Commercialization

For the commercial livestock application of cloning by nuclear transfer, additional technologies must be developed. *In vitro* matured oocytes must be used as recipient oocytes, it must be possible to develop the resulting embryo *in vitro* to a stage that can be nonsurgically transferred to a recipient, and it must be possible to preserve the embryos by freezing. The pig is at a disadvantage as compared with the sheep and cow, since pig embryos are less tolerant to freeze preservation (50). In addition, only recently have methods been presented that result in normal *in vitro* development (51, 52) and offspring following nonsurgical embryo transfer (53).

An additional requirement for commercial application is a market. This could be developed after the uniformity of an individual clonal line is established. A company or producer could market a clonal line of embryos that would have a guaranteed birthing ease, neonatal growth rate, postweaning growth rate, behavioral characteristics, disease resistance, marbling characteristics, and size and shape of various meat cuts. As the phenotype of the clonal line is established and tested, these parameters would be defined as well as the ration and environment to achieve these parameters.

Future Direction

Future directions should focus mainly upon making the procedures more efficient, defining the impact of the genetic contributions, and defining the biochemical reprogramming that occurs to transferred nuclei. The current source of donor nuclei is from early stage embryos; however, a possible additional source of donor nuclei may be from embryonic stem cells. These cells have been isolated from mouse embryos, they can be cultured *in vitro* to large numbers, and, most important, they can be chimerized with the inner cell mass of blastocyst stage embryos. The embryonic stem cells that are incorporated into the fetus are stabile and can contribute to the germ line (reviewed by Prather *et al.* (54)). Since these are a relatively undifferentiated cell type, they are good candidates for a source of nuclei for the nuclear transfer. Since these cells can be transformed *in vitro*, lines of transgenic animals are a distinct possibility.

The clonal technology has a very bright future. Currently, the various steps in the procedure need not only to be refined, but in some cases defined. The procedures are inefficient, but they do work and it is now our opportunity to evaluate the parameters that affect development after nuclear transfer to make the overall procedure more efficient.

This manuscript was prepared while supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement 88-37240-4197 and Food for the Twenty-first Century, and is a contribution from the Missouri Agricultural Experiment Station Journal Series 11,173.

1. Spemann H. Embryonic Development and Induction. New York: Hafner Publishing Co., pp210–211, 1938.
2. Briggs R, King TJ. Transplantation of living cell nuclei from blastula cells into enucleated frogs' eggs. Proc Natl Acad Sci USA 38:455–463, 1952.
3. Willadsen SM. Nuclear transplantation in sheep embryos. Nature 320:63–65, 1986.
4. Prather RS, Barnes FL, Robl JM, First NL. Multiplication of bovine embryos. Biol Reprod 34(Suppl 1):192, 1986.
5. McGrath J, Solter D. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220:1300–1302, 1983.
6. Prather RS, First NL. The cloning of embryos. J Reprod Fertil [Suppl] 40:227–234, 1990.
7. Berg H. Electrofusion of blastomeres and blastocysts of mouse embryos. Bioelectricity Bioenerg 9:223–228, 1982.
8. Zimmerman U, Vienken J. Electric field-induced cell-to-cell fusion. J Membr Biol 67:165–182, 1982.
9. Gurdon JB. The transplantation of living cell nuclei. Adv Morphog 4:1–43, 1964.
10. Gurdon JB, Brown DD. Cytoplasmic regulation of RNA synthesis and nucleolus formation in developing embryos of *Xenopus laevis*. J Mol Biol 12:27–35, 1965.
11. Szollosi D, Czolowska R, Szollosi MS, Tarkowski AK. Remodeling of mouse thymocyte nuclei depends on the time of their transfer into activated, homologous oocytes. J Cell Sci 91:603–613, 1988.
12. Czolowska R, Modlinski JA, Tarkowski AK. Behavior of thymocyte nuclei in nonactivated and activated mouse oocytes. J Cell Sci 69:19–34, 1984.
13. Stice SL, Robl JM. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol Reprod 39: 657–664, 1988.
14. Prather RS, Sims MM, First NL. Nuclear transplantation in the pig embryo: Nuclear swelling. J Exp Zool (in press).
15. Collas P, Robl JM. Age of recipient oocyte for nuclear transplantation in the rabbit. Biol Reprod 40(suppl 1):18, 1989.
16. Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B, First NL. Nuclear transplantation in bovine embryos. J Anim Sci 64:642–647, 1987.

17. McGrath J, Solter D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development *in vitro*. Science 226:1317–1319.
18. DiBerardino MA, Hoffner NJ. Nucleocytoplasmic exchange of nonhistone proteins in amphibian embryos. Exp Cell Res 94:235–252, 1975.
19. Leonard RA, Hoffner NJ, DiBerardino MA. Induction of DNA synthesis in amphibian erythroid nuclei in *Rana* eggs following conditioning in meiotic oocytes. Dev Biol 92: 343–355, 1982.
20. Merriam RW. Movement of cytoplasmic proteins into nuclei induced to enlarge and initiate DNA or RNA synthesis. J Cell Sci 5:333–349, 1969.
21. Feldher CM, Pomerantz J. Mechanism for the selection of nuclear polypeptides in *Xenopus* oocytes. J Cell Biol 78:168–175, 1978.
22. Prather RS, Sims MM, Maul GG, First NL, Schatten G. Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis. Biol Reprod 41:123–132, 1989.
23. Prather RS, Kubiak J, Maul GG, First NL, Schatten G. The association of nuclear lamins A and C is regulated by the developmental stage of the mouse oocyte or embryonic cytoplasm. J Exp Zool (in press).
24. Borer RA, Lehner CF, Eppenberger HM, Nigg EA. Major nucleolar protein shuttle between nucleus and cytoplasm. Cell 56:379–390, 1989.
25. DiBerardino MA, Hoffner N. Origin of chromosomal abnormalities in nuclear transplants—A reevaluation of nuclear differentiation and nuclear equivalence in amphibians. Dev Biol 23:185–209, 1970.
26. Gurdon JB. Nuclear transplantation in eggs and oocytes. J Cell Sci [Suppl] 4:287–318, 1986.
27. DiBerardino MA. Nuclear and chromosomal behavior in amphibian nuclear transplants. Int Rev Cytol 9:129–160, 1979.
28. Bolton VN, Oades PJ, Johnson MH. The relationship between cleavage, DNA replication and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol 79:139–169, 1984.
29. Tomaneck M, Kopecny V, Kanka J. Genome reactivation in developing early pig embryos: An ultrastructural and autoradiographic analysis. Anat Embryol 180:309–316, 1989.
30. Szollosi D. Nucleolar transformation and ribosome development during embryogenesis of the rat. J Cell Biol 31:115a, 1966.
31. Camous S, Kopecny V, Flechon J-E. Autoradiographic detection of the earliest stage of [^{3}H]uridine incorporation into the cow embryo. Biol Cell 58:195–200, 1986.
32. Van Blerkom J, McGaughey R. Molecular differentiation of the rabbit ovum. II During the preimplantation development of *in vivo* and *in vitro* matured oocytes. Dev Biol 63:151–164, 1978.
33. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 82:769–775, 1988.
34. Smith LC, Wilmut I. Influence of nuclear and cytoplasmic activity on the development *in vivo* of sheep embryos after nuclear transplantation. Biol Reprod 40: 1027–1035, 1989.
35. Gurdon JB, Brennan S, Fairman S, Mohun TJ. Transcription of muscle-specific actin genes in early *Xenopus* development: nuclear transplantation and cell dissociation. Cell 38:691–700, 1984.
36. Wakefield L, Gurdon JB. Cytoplasmic regulation of 5S RNA genes in nuclear-transplant embryos. EMBO J 2:1613–1619, 1983.
37. Prather RS, Sims MM, First NL. Nuclear transplantation in early pig embryos. Biol Reprod 41:414–418, 1989.
38. Prather RS, First NL. Cloning embryos by nuclear transfer. J Reprod Fertil [Suppl] (in press).
39. Bondioli KR, Westhusin ME, Looney CR. Production of identical bovine offspring by nuclear transfer. Theriogenology 33:165–174, 1990.
40. Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: Assessment of donor nuclei and recipient oocyte. Biol Reprod 37:859–866, 1987.
41. Alt FW, Blackwell K, Yancopoulos GD. Development of primary antibody repertoire. Science 238:1079–1087, 1987.
42. Olivo PD, Van de Walle MJ, Laipis PJ, Hauswirth WW. Nucleotide sequence evidence for rapid genotype shifts in the bovine mitochondrial DNA D-loop. Nature 306:400–40, 1983.
43. Hartl F-U, Neupert W. Protein sorting to mitochondria: evolutionary conservations of folding and assembly. Science 247:930–938, 1990.
44. Hall JL, Ramanis Z, Luck DJL. Basal body/centriolar DNA: Molecular genetic studies in *chlamydomonas*. Cell 59:121–132, 1989.
45. Szollosi D, Calarco P, Donahue RP. Absence of centrioles in the first and second meiotic spindles of mouse oocytes. J Cell Sci 11:521–541, 1972.
46. Schatten G, Simerly C, Schatten H. Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc Natl Acad Sci USA 82:4152–4156, 1985.
47. Seidel GE Jr. Are identical twins produced from micromanipulation always identical? Proc Ann Conf Artific Insem and Embryo Transfer in Beef Cattle (Denver). Natl Assoc Anim Breeders, Columbia, MO pp 50–53, 1985.
48. Seidel GE Jr. Production of genetically identical sets of mammals: Cloning? J Exp Zool 228:347–354, 1983.
49. Biggers JD. The potential use of artificially produced monozygotic twins for comparative experiments. Theriogenology 26:1–25, 1986.
50. Hayashi S, Kobayashi K, Mizuno J, Saitoh K, Hirano S. Birth of piglets from frozen embryos. Vet Rec 125:43–44, 1989.
51. Hagen DR, Prather RS, Sims MM, First NL. Development of pig embryos to blastocysts (BL) in sheep and pig oviducts, and coculture *in vitro*. J Anim Sci 67(suppl 1):518, 1989.
52. Beckmann LS, Cantley TC, Rieke AR, Day BN. Development and viability of one- and two-cell porcine embryos cultured through the "four-cell block." Theriogenology 33:193, 1990.
53. Sims MM, First NL. Nonsurgical embryo transfer in swine. J Anim Sci 65(suppl 1):386, 1987.
54. Prather RS, Hagemann LJ, First NL. Preimplantation mammalian aggregation and injection chimeras. Gamete Res 22:233–247.

BIOLOGY OF REPRODUCTION 60, 998–1005 (1999)

Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells[1]

David N. Wells,[2] Pavla M. Misica, and H. Robin Tervit

AgResearch, Ruakura Research Centre, PB 3123, Hamilton, New Zealand

ABSTRACT

Adult somatic cell nuclear transfer was used to determine the totipotent potential of cultured mural granulosa cells, obtained from a Friesian dairy cow of high genetic merit. Nuclei were exposed to oocyte cytoplasm for prolonged periods by electrically fusing quiescent cultured cells to enucleated metaphase II cytoplasts 4–6 h before activation (fusion before activation [FBA] treatment). Additionally, some first-generation morulae were recloned by fusing blastomeres to S-phase cytoplasts. A significantly higher proportion of fused embryos developed in vitro to grade 1–2 blastocysts on Day 7 with FBA (27.5 ± 2.5%) than with recloning (13.0 ± 3.6%; $p < 0.05$). After the transfer of 100 blastocysts from the FBA treatment, survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. Ten heifer calves were delivered by elective cesarean section; all have survived. After the transfer of 16 recloned blastocysts, embryo survival on Day 60 was 38%; however, no fetuses survived to Day 100. DNA analyses confirmed that the calves are all genetically identical to the donor cow. It is suggested that the losses throughout gestation may in part be due to placental dysfunction at specific stages. The next advance in this technology will be to introduce specific genetic modifications of biomedical or agricultural interest.

INTRODUCTION

There is intense scientific interest in the field of somatic cell nuclear transfer, principally to enable both the multiplication of elite livestock and the engineering of transgenic animals, for various agricultural and biomedical purposes. The realization of these applications involves the development of embryo and cell manipulation techniques that facilitate the totipotent potential of cultured and/or differentiated cell nuclei to be expressed.

The successful production of offspring derived after nuclear transfer depends upon a wide variety of factors. One factor of importance is appropriate cell cycle coordination. Although there are no definitive reports comparing the effects of cell cycle synchrony between donor nuclei from cultured somatic cells and recipient cytoplasts, there currently appear to be at least two methods that are successful in yielding viable cloned offspring. The first uses donor cells in a quiescent state, in which the cells are presumed to have exited the normal cell cycle and have arrested in what is termed G0. This may be induced in cultured cells by, for example, serum starvation [1, 2] or by using cells that are naturally arrested in this state directly from the animal [3, 4]. It is possible that the reduction in transcriptional activity and chromatin modification associated with cells in G0 [5] may facilitate the reprogramming of nuclei following exposure to oocyte cytoplasmic factors, enabling normal development to occur in some instances [1, 2]. The failure of earlier studies in amphibians to generate viable clones from adults after the use of G0 cells (e.g., [6–8]) may have been due to other limiting facets of the nuclear transfer technique and/or the greater difficulty (or impossibility) to reprogram the nuclei of some terminally differentiated cell types. The latter phenomenon may be the case with Sertoli and neuronal cells in the mouse [3].

The second method that appears successful in somatic cell nuclear transfer involves exploiting the various factors present in the cytoplasm of the metaphase-arrested oocyte that may facilitate the remodeling and reprogramming of somatic cell nuclei [9]. In addition, prolonged exposure to this cytoplasmic environment may aid this process further and appears to have conferred nuclear totipotency to nonquiescent cells [10]. This prolonged cytoplasmic exposure may be achieved by fusing cells before the activation of the reconstructed embryo [9, 11–13]. However, for it to be successful and to avoid chromosomal damage and abnormal ploidy in the resulting embryos, the cell cycle stage of the donor nucleus must be compatible with the high levels of maturation-promoting factor present in the metaphase II (MII) oocyte [14, 15]. For these reasons, only nuclei that have a diploid (2C) DNA content (that is, in either G1 [16] or G0 of the cell cycle [1–3]), or in metaphase [17]) are compatible with nuclear transplantation to enucleated MII oocytes. Although no effect of prolonged exposure of quiescent cells to the MII cytoplasm has been observed in sheep [1], effects have been seen in other species. With unsynchronized bovine cultures of undifferentiated embryonic cells, in which the majority of cells were reported to be in G1, exposure to MII cytoplasm for 4 h before activation significantly increased development to blastocyst, compared to embryos reconstructed by either simultaneous fusion and activation, or with preactivated cytoplasts [18]. Although fetal development did not proceed beyond Day 55 in this study [18], the strategy of fusion before activation has resulted in viable cloned calves using fetal fibroblasts in another study, in which once again the majority of cells were reported to have been in G1 [10]. With quiescent bovine cells derived from either the fetus [12] or adult [13], embryo development was also significantly increased by prolonging the period of exposure of the nucleus to the MII cytoplasm before activation. Similarly in the mouse, embryo and fetal development were both improved with exposure of cumulus cells in G1/G0 to MII cytoplasm for 1–6 h [3].

Although it is now clearly possible to produce cloned offspring from differentiated mammalian cells after nuclear transfer, the overall success rate is currently low in the above-mentioned studies (0.4–1.8%). Furthermore, in most studies, the exact stage of the cell cycle of the successful donor cells, which ultimately yielded the viable cloned offspring, remains uncertain.

The principal objective of the nuclear transfer studies

Accepted December 3, 1998.
Received October 22, 1998.
[1]This research was supported by the New Zealand Foundation for Research, Science, and Technology (contract number C10 402).
[2]Correspondence: David Wells, AgResearch, Ruakura Research Centre, East Street, PB 3123, Hamilton, New Zealand. FAX: 64 7 8385536; e-mail: wellsd@agresearch.cri.nz

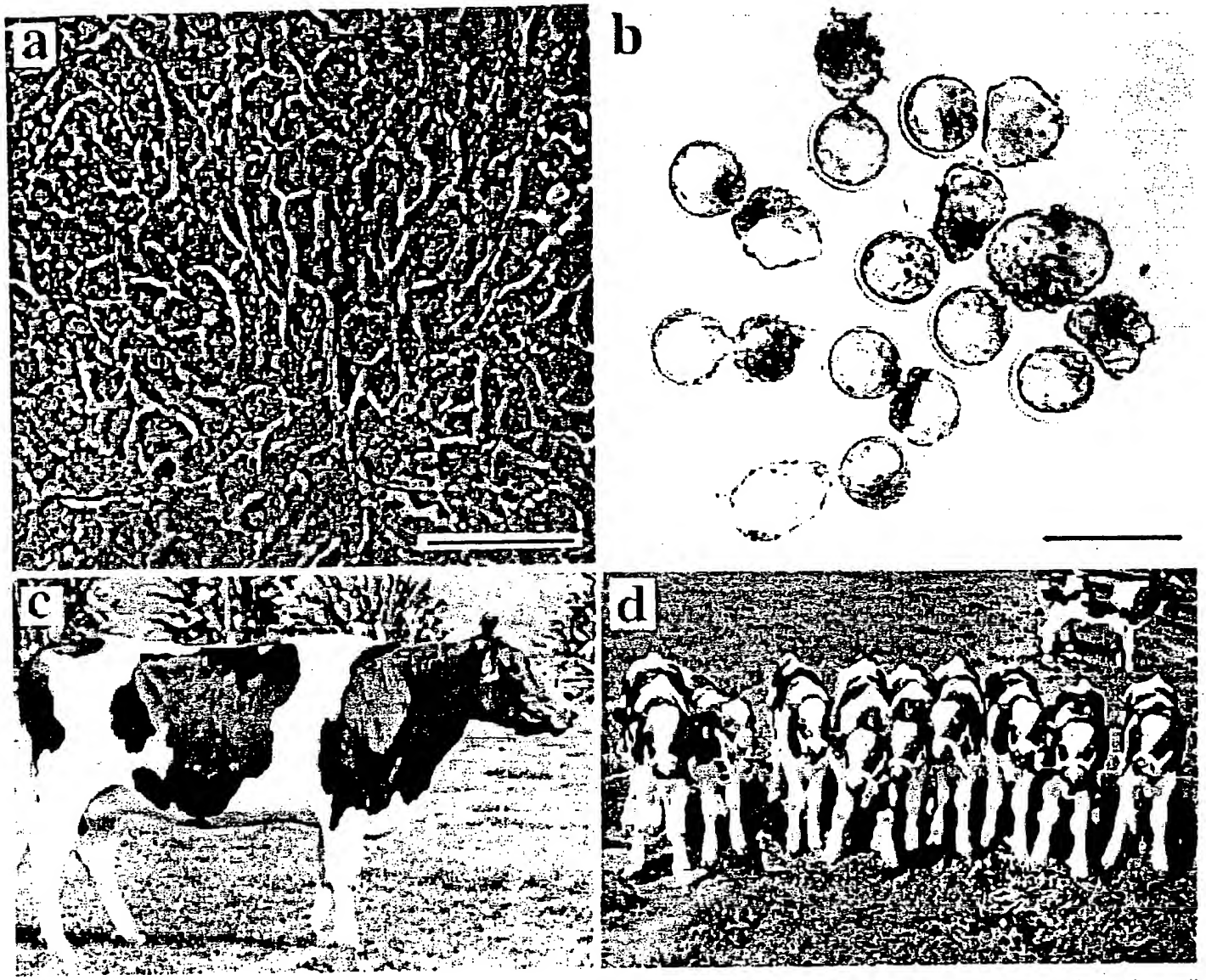


FIG. 1. Demonstration of the totipotency of adult bovine mural granulosa cells after nuclear transfer. a) Morphology of the EFC mural granulosa cells at passage five. Bar = 100 μm. b) Hatching blastocysts produced seven days postfusion from the FBA treatment with EFC cells. Bar = 200 μm. c) The Friesian donor cow. d) Ten cloned calves genetically identical to the cow shown in c. Insert shows one calf recovering from a broken leg at the time of submission.

described here was to determine the nuclear totipotency of cultured adult bovine mural granulosa cells. We also investigated the effect on embryo and fetal development of prolonged exposure of the transferred nucleus to the oocyte cytoplasm by either fusing quiescent granulosa cells before activation or by recloning the first-generation nuclear transfer embryos. Recloning provides an additional method of allowing a longer period for nuclear reprogramming to occur and has improved development in amphibians [9]. During the course of these experiments, we have established the efficiency of cloning adult females using mural granulosa cells and have begun to identify the important areas of future research needed to improve the success rates beyond the current 10% embryo survival to term reported here.

MATERIALS AND METHODS

Isolation of Mural Granulosa Cells

A primary cell line (EFC) was established from mural granulosa cells collected by aspirating the ovarian antral follicles (3–10 mm in diameter) from a three-year-old Friesian dairy cow of high genetic merit (Fig. 1c), using an ultrasound-guided, transvaginal probe [19]. The collected cells were centrifuged and washed once in culture medium before seeding onto a four-well tissue culture plate (Nunclon, Roskilde, The Netherlands). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium (Life Technologies, Auckland, New Zealand) supplemented with 10% v:v fetal calf serum (FCS; Life Technologies) and sodium pyruvate to a final concentration of 1 mM. During the first seven days, penicillin, streptomycin, and amphotericin B were added. The cell line was routinely passaged using an enzymatic solution (TEG) comprising 0.25% w:v trypsin (porcine pancreas; Life Technologies) and 0.04% w:v EGTA (Sigma, St. Louis, MO) for 7 min at 39°C. Small aliquots of early-passage EFC cells were frozen in 10% dimethyl sulfoxide (BDH, Poole, Dorset, England). The average cell population doubling time was 42.0 ± 1.1 h and the cells were maintained for at least nine passages in culture, representing approximately a total of

15 cell population doublings. The cell morphology is illustrated in Figure 1a. All cells used for nuclear transfer in these experiments had been previously frozen and thawed.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from mature cows, placed in saline (30°C), and transported within 2 h to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3–6-mm follicles using an 18-gauge needle and negative pressure (40–50 mm Hg). COCs were collected into HEPES-buffered tissue culture medium 199 (H199; Life Technologies) supplemented with 50 μg/ml heparin (Sigma) and 0.4% w:v BSA (Immuno-Chemical Products [ICP], Auckland, New Zealand). Before in vitro maturation, only those COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered tissue culture medium 199 + 10% FCS. Ten COCs were transferred in 10 μl of this medium and placed into a 40-μl drop of maturation medium in 5-cm Petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised tissue culture medium 199 supplemented with 10% FCS, 10 μg/ml ovine FSH (Ovagen; ICP), 1 μg/ml ovine LH (ICP), 1 μg/ml estradiol (Sigma), and 0.1 mM cysteamine (Sigma) [20]. Microdrop dishes were cultured at 39°C in a humidified 5% CO_2 in air atmosphere for 20 h. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered synthetic oviduct fluid (HSOF) [21] for 3 min; this was followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Granulosa Cells

Media. With embryos that were reconstructed by fusing donor cells and MII cytoplasts before activation (fusion before activation [FBA] treatment), matured oocytes, cytoplasts, and reconstructed embryos were either held or manipulated in HSOF- or SOF-based medium (as appropriate) without calcium for the period following maturation and until 30 min before activation. After this point, calcium was present in all media formulations used.

Enucleation. Oocytes matured for 20–22 h were enucleated with a 30-μm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were previously stained in HSOF medium containing 10% FCS, 5 μg/ml Hoechst 33342, and 7.5 μg/ml cytochalasin B (Sigma) for 20 min. Enucleation was confirmed by visualizing the karyoplast, while still inside the pipette, under ultraviolet light. After enucleation, the resulting cytoplasts were washed extensively in HSOF + 10% FCS and were held in this medium until injection of donor cells.

Preparation of cells. Donor cells were used for nuclear transfer between passages three and eight of culture. EFC cells were induced to enter a period of quiescence (presumptive G0) by serum deprivation [1]. One day after routine passage, the culture medium was aspirated, and the cells were washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The cells were returned to culture for a further 8–18 days before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in HSOF + 0.5% FCS and remained in this medium until injection.

Microinjection. Recipient cytoplasts were dehydrated in HSOF containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A 35-μm pipette (external diameter) containing the donor cell was introduced through the same slit in the zona pellucida as made during enucleation, and the cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After injection, the reconstructed embryos were rehydrated in two steps; first in HSOF containing 10% FCS and 2.5% sucrose for 5 min and then in HSOF + 10% FCS until fusion.

Cell fusion. Reconstructed embryos in the FBA group were electrically fused at 24 h poststart of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES, and 0.05% fatty acid-free (FAF) BSA (Sigma) with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 μm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 2.25 kV/cm for 15 μsec each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). After the electrical stimulus, the reconstructed embryos were washed in HSOF + 10% FCS. They were then checked for fusion by microscopic examination.

Activation. After fusion, FBA embryos were cultured for a period of 4–6 h in SOF + 10% FCS before chemical activation. Thirty minutes before activation, fused embryos were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30-μl drops of 5 μM ionomycin (Sigma) in HSOF + 1 mg/ml FAF BSA for 4 min at 37°C. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 min before culture for 4 h in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) in SOF + 10% FCS.

Recloning: Nuclear Transfer with Embryonic Blastomeres

Unlike the FBA treatment, all medium formulations contained calcium. After enucleation, cytoplasts were preactivated at 24 hpm with ionomycin and 6-DMAP, as described above. Donor blastomeres were prepared from compacting morulae 5 days after the fusion of quiescent granulosa cells. Zonae pellucidae were digested using 0.5% w:v pronase (Sigma) for approximately 30 sec. Blastomeres were separated by incubation for 30 min in calcium- and magnesium-free PBS containing 7.5 μg/ml cytochalasin B, with the aid of gentle pipetting. Blastomeres were transferred to the injection chamber in a drop of medium containing cytochalasin B and were individually injected into the perivitelline space of dehydrated cytoplasts around 6—8 h after the activation stimulus. Cell fusion was induced with two DC pulses of 1.20 kV/cm for 80 μsec each in the fusion buffer described above. Successfully fused embryos were then placed into culture as described below.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20-μl drops of SOFaaBSA (8 mg/ml FAF BSA; Sigma) [22] overlaid with paraffin oil. Whenever possible, groups of five to six embryos were cultured together. Embryos were cultured in a

humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39°C in a 5% CO_2:7% O_2:88% N_2 gas mix. On Day 5, embryos were transferred to fresh 20-μl drops of SOFaaBSA + 10% charcoal-stripped FCS (csFCS) [23]. On Day 7 postfusion, development to morulae and blastocysts was recorded, and embryos were morphologically assessed using a subjective grading system based on a scale of 1–4, inclusive, representing embryos ranging from excellent to poor quality, respectively. The cell number of some embryos was determined by counting stained nuclei, using an established image analysis method described previously [24].

Oocyte Activation Controls

Oocytes matured for 20 h were stripped of cumulus cells, and those having the first polar body were selected. Before activation at the appropriate time, oocytes were held in calcium-free medium. Oocytes were activated with ionomycin at either 24 or 30 hpm and then cultured in SOF (containing calcium) + 10% FCS and 6-DMAP, as described above for nuclear transfer embryos. After a 4-h incubation, oocytes were washed and transferred to SOF (containing calcium) + 10% FCS until they were whole-mounted and fixed, between 6 and 12 h postactivation, using methods described elsewhere [25]. After staining with 1% orcein in 45% acetic acid, all oocytes were examined by phase-contrast microscopy for the presence of pronuclei formation.

As a negative control for electrical activation at 24 hpm, oocytes were exposed to the electrical stimulation used for fusion as described above. Oocytes were then cultured in SOF + 10% FCS without calcium, exactly as used for the FBA nuclear transfer embryos, but for 10–12 h. Control oocytes were then mounted and fixed, before subsequent examination of chromatin configuration to identify oocytes in either anaphase II or telophase II, or with pronuclei, as evidence of activation.

Embryo Transfer

Recipient cows were synchronized by a single 10-day CIDR-plus (InterAg, Hamilton, New Zealand) treatment. Six days after CIDR-plus insertion, each cow received 250 mg chlorprostenol (1 ml estrumate; Schering-Plough, Union, NJ). The mean onset of estrus was observed 48 h after CIDR-plus withdrawal. Embryo transfer was performed nonsurgically on Day 7 after estrus (estrus = Day 0 = day of fusion). Each cow received two blastocysts of grade 1–2 quality, transferred in HSOF + 5% csFCS medium into the uterine lumen ipsilateral to the corpus luteum.

Determination of Embryonic Survival and Calving

All cows were examined by ultrasonography (Piemed 200 scanner, with a linear 3.5–5 MHz rectal probe; Philipsweg, Maastricht, The Netherlands) on Day 60 of gestation to record fetal development. Pregnant cows were monitored by rectal palpation at regular intervals thereafter. Commencing approximately 2 wk before expected full term, pregnant cows were monitored daily by both rectal and vaginal examination to determine fetal position and cervical dilation. Parturition was induced with an injection of 20 mg dexamethasone (Dexadreson; Intervet, Boxmeer, The Netherlands) administered 17 h before cesarean section between Days 276 and 281.

Neonatal Care

Immediately after delivery, the newborn calves were weighed and transported to a room maintained at 25°C for neonatal monitoring. The calves were dried, rectal temperature was taken, oxygen therapy was provided via a face mask, and the calves were positioned in sternal recumbency. Oxygen therapy was discontinued typically after 30 min, but this depended upon an assessment of general calf demeanor and supplementary analytical data. Blood gas and electrolyte values were determined on some calves by immediately analyzing samples of anaerobic, heparinized blood obtained from the brachial artery, using an i-STAT clinical analyzer (Sensor Devices, Waukesha, WI). The time taken for the calves to stand unaided was recorded, and colostrum was offered via a nipple bottle at this point. It was ensured that calves received a volume of colostrum equivalent to 10% of their live weight in the first 10 h. After their first feed, calves were moved to a recovery room set at 18°C overnight and were then subsequently reared outdoors. All calves received either Zaquilan (Schering-Plough) or Excenel (Upjohn, Kalamazoo, MI) as a precaution against respiratory infection. Jugular venous blood samples were taken 18 h after delivery for routine biochemistry and hematology analyses, and at regular intervals thereafter.

Microsatellite Analyses

Genomic DNA was extracted from the white blood cells collected from the nuclear transfer-derived calves and recipient cows using a guanidine hydrochloride method [26]. Sixteen microsatellite markers (see *Results* for specific loci) were analyzed using methods described elsewhere [27]. The resulting microsatellite alleles for the nuclear transfer-derived calves were compared with those from the donor cow from which the mural granulosa cells were obtained, and contrasted with those from the recipient cows that carried the respective pregnancies.

Animal Ethics

This project was approved by both the AgResearch Ruakura Animal Ethics Committee and the AgResearch Ruakura Biosafety Committee.

Statistical Analyses

The proportional data for cell fusion, in vitro development of embryos, and subsequent survival following embryo transfer were all analyzed by fitting generalized linear models using binomial distributions within the GENSTAT 5 statistical package (Lawes Agricultural Trust, Rothamsted, UK). Embryo cell numbers were analyzed after log-transformation.

RESULTS

Embryo Development

The fusion of embryonic blastomeres to cytoplasts in the recloned group was significantly higher than the fusion of quiescent granulosa cells in the FBA treatment (88.7 ± 3.8% vs. 77.4 ± 2.2%, $p < 0.05$) (Table 1). Electro-fusion with granulosa cells was not affected by either the length of time the cells were in low serum (8–18 days) or passage number (3–8).

Although there was no difference in the proportion of

TABLE 1. Effect of nuclear transfer treatment on the electrical cell fusion rates and the proportion of fused embryos developing to morulae or blastocysts by Day 7.

Treatment	Fusion	Number cultured	Blastocysts (grade 1–2)	Blastocysts (grade 1–3)	Total morulae & blastocysts
FBA	77.4%[a]	552	152 (27.5%)[c]	282 (51.1%)[e]	383 (69.4%)
Recloned	88.7%[b]	146	19 (13.0%)[d]	51 (34.9%)[f]	84 (57.5%)

[ab], [cd], [ef], $p < 0.05$.

fused embryos that developed to the morula or blastocyst stage (grades 1–4) by Day 7, significantly more FBA embryos developed into both grade 1–2 and grade 1–3 blastocysts (27.5 ± 2.5%; 51.1 ± 2.2%) compared to the recloned embryos (13.0 ± 3.6%; 34.9 ± 4.0%, respectively; $p < 0.05$) (Table 1; Fig. 1b). Within the FBA treatment, there was no effect of either granulosa cell passage number or length of time in low serum, on subsequent embryo development rates.

Embryo Cell Numbers

Embryo cell number within each embryo stage and grade category was not affected by FBA or recloning treatments, nor by length of time in low serum or passage number of the granulosa cells. The average cell number for grade 1–2 and grade 3 blastocyst-stage embryos was 132 ± 14 (n = 58) and 82 ± 3 cells (n = 166; $p < 0.05$), respectively. For those embryos that had only developed to morulae or early blastocysts seven days postfusion, the average cell number (26 ± 2) was significantly less ($p < 0.001$; n = 84).

Activation of Control Oocytes

In the FBA and recloning treatments, both the reconstructed embryos and the cytoplasts, respectively, were artificially activated, using ionomycin and 6-DMAP. However, the relative age of the cytoplasm differed, with activation occurring at 24 hpm in the recloning treatment and at 30 hpm with the FBA embryos. Studies with control MII oocytes showed that the activation rate, as observed by pronuclear formation, was not different at either 24 hpm (95.9 ± 1.6%; n = 165) or 30 hpm (93.1 ± 3.4%; n = 131).

With the experimental conditions used here to achieve cell fusion at 24 hpm in the FBA treatment, only 1.0% (n = 97) of control MII oocytes were electrically activated by the stimulus.

Embryo Survival

A total of 100 grade 1–2 blastocyst-stage embryos from the FBA treatment were transferred to 50 recipient cows. Survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. In contrast, of the 16 embryos transferred from the recloned group, 38% resulted in fetuses present at Day 60; however, none survived to Day 100 of pregnancy. In the FBA treatment, seven fetuses were lost in the third trimester, including three sets of twin pregnancies. These losses resulted from an excessive accumulation of allantoic fluid. Ten heifer calves were delivered by cesarean section between Days 276 and 281 from eight recipient cows. All 10 cloned calves have survived (Fig. 1d). After the first few hours of life, regular animal health tests showed that the calves were physiologically healthy. The calves were derived from mural granulosa cells at either passage three or five of culture. There was no effect of length of serum deprivation or passage number on embryo survival rates.

Postnatal Characteristics of Cloned Calves

The average birth weights of the four twin calves and the six singles were 30.1 ± 2.1 kg and 44.1 ± 2.1 kg, respectively (range: 26.5–51.0 kg). After cesarean section, the average time for the majority of calves (8 of 10) to stand unaided was 39 min (range 30–50 min). However, two larger calves with birth weights of 49 and 51 kg required 90 and 120 min, respectively. All calves had a strong suckling reflex and, once standing, all drank colostrum from the bottle. In the case of one calf, 1 mg epinephrine and 20 mg doxapram were administered within 5 min of delivery in order to stimulate the cardiac and respiratory systems, respectively. This calf responded well to treatment and was standing and feeding 40 min later. The average rectal temperature within 10 min of delivery was 39.7 ± 0.1°C. However, by 1 h and 8 h postdelivery, rectal temperatures had fallen to 38.9 ± 0.2°C and 38.2 ± 0.1°C, respectively. Plasma bicarbonate was generally normal within the first hour (25.7 ± 0.8 mM; n = 3); however, one calf had metabolic acidosis over this period (16 mM HCO^{3-}). Of the five calves examined, four had normal plasma glucose levels (3.6 ± 0.5 mM); however, one calf was hypoglycemic after 90 min (1.4 mM glucose) and took several hours before it stabilized to normal levels. While all the calves were viable, a number of abnormalities were noted in the placentas of four recipient cows, which included cases of enlarged umbilical vessels, edematous membranes, and greater than usual allantoic fluid volume. None of these abnormalities appeared to compromise fetal development.

Microsatellite Analyses

Microsatellite DNA analyses examining 16 loci confirm that all the calves are genetically identical to the donor cow from which the granulosa cells used for nuclear transfer were obtained. Additionally, the cloned calves are not genetically related to the respective recipient cows (Fig. 2; a–d represent microsatellite markers texan10, bms1789, bm711, and bms941, respectively). In all of the autoradiograms, lane 1 represents the donor cow and lanes 2–11 the adult cloned calves, with lanes 12–19 representing the eight recipient cows. The other microsatellite markers examined were agla232, bmc4214, bms1353, bms2614, cssm38, oarfcb20, rm216, rm327, rm737, tgla122, tgla126, and tgla227 (data not shown).

DISCUSSION

We have shown that mural granulosa cells obtained from a living adult cow can be reprogrammed by nuclear transfer and result in the production of viable cloned calves. Exposure of the quiescent nucleus to cytoplasmic factors present in the MII oocyte for 4–6 h resulted in relatively high

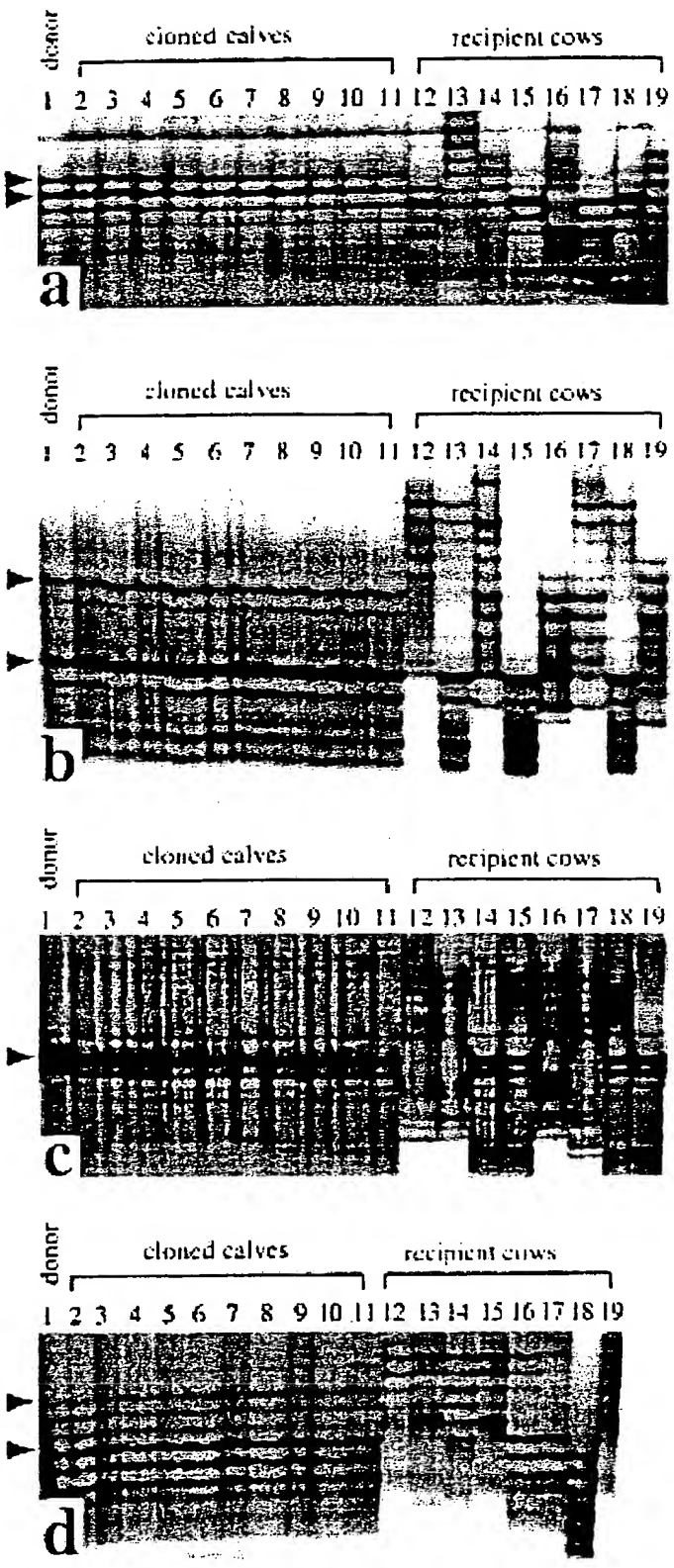


FIG. 2. Autoradiograms demonstrating the genetic origin of the cloned calves using microsatellite DNA markers texan10, bms1789, bm711, and bms941, in a, b, c, and d, respectively (see text for details).

rates of development to transferable-quality embryos in vitro (28%) and survival to term (10%). Collectively these data suggest improved reprogramming of the somatic cell nucleus. However, the technique remains limited by the substantial fetal loss that occurs throughout gestation and the abnormalities associated particularly with placentation and parturition.

It has not yet been determined which cell types from the adult animal are the most successful for somatic cell nuclear transfer. Earlier reports have shown that quiescent cells from the mammary gland in the sheep [2] and the cumulus cells surrounding the ovulated mouse oocyte [3] are both successful cell types, with overall efficiencies (in terms of live animals from successfully fused or injected cytoplasts) of 0.4% and 0.9%, respectively. In comparison, with the bovine mural granulosa cells used here for nuclear transfer, we report an overall efficiency of 2.8%, from the in vitro development and subsequent transfer of grade 1 and 2 blastocysts only (we have no data on the viability of grade 3 blastocysts). Mural granulosa cells differ in both their function and fate from cumulus granulosa cells [28]. Since the EFC cells were isolated before terminal differentiation into luteal cells, it is probable that the mural granulosa cells used here were less differentiated than expanded and mucified cumulus granulosa cells obtained from ovulated oocytes [4]. In an earlier study, bovine cumulus granulosa cells failed to produce pregnancies after nuclear transfer [29]; however, this cell type has recently resulted in the birth of adult clones in the mouse [3]. We suggest that mural granulosa cells will prove to be a suitable cell type from which to clone genetically elite cows because of the ease of repeated and noninvasive collection of cells using standard "ovum pick-up" techniques [19].

It has been shown in amphibians that the efficiency of nuclear transfer, represented as the proportion of clones developing normally, decreases as nuclei from more differentiated cell types, or more advanced developmental stages, are used [30]. Thus it had been postulated that as development proceeds, the totipotency of nuclei becomes restricted. With sheep and cattle in our laboratory, at least, this effect has not been observed. The proportion of fused embryos that develop to blastocysts (grade 1–3) from embryonic [31, 32], fetal [12], and adult (this study) cell types are 26%, 52%, and 51%, respectively. The corresponding production of viable offspring from embryos transferred in each of these categories is 5%, 11%, and 10%, respectively. Interestingly, these efficiencies with cultured somatic cells are not dissimilar to those achieved in some studies after nuclear transfer with embryonic blastomeres [33, 34]. By way of comparison, the efficiency of standard in vitro production (IVP) of bovine embryos in our laboratory is typically 40% development to blastocyst, with 39% of transferred embryos surviving to term [23]. Significantly, the quality of adult cloned embryos as determined by the cell number of grade 1 and 2 blastocysts on Day 7 is not different from that of bovine IVP embryos [23]. Thus the proportion and quality of blastocysts that develop are similar after in vitro fertilization and somatic cell nuclear transfer; however, the development to term of cloned embryos is currently only one-quarter that of IVP.

All ten nuclear transfer-derived calves reported here resulted from the transfer of embryos produced after the fusion of quiescent mural granulosa cells with enucleated MII oocytes, which were then activated to commence development 4–6 h later. We have previously demonstrated that embryo development is significantly increased by fusing quiescent donor cells with metaphase II cytoplasm before activation (FBA treatment), in comparison to simultaneous fusion and activation at either 24 or 30 hpm with either bovine fetal fibroblasts [12] or adult mural granulosa cells [13]. The prolonged exposure of transferred nuclei to oo-

cyte cytoplasmic factors possibly facilitates nuclear remodeling and reprogramming, as suggested previously [9, 35]. Improved embryo development with the FBA treatment, compared to simultaneous fusion and activation, has also been observed with unsynchronized cultures of bovine embryonic cells [18] and with mouse cumulus cells [3], in which the majority of cells were reported to be either in G1 or in a natural G0/G1 stage of the cell cycle, respectively. In addition to the effect of FBA, there may be benefits in synchronizing cells in G0, since blastocyst rates in the bovine species with unsynchronized embryonic [18] or fetal [10] cells have only been 10–12% compared to 51–52% reported with both quiescent adult and fetal cells (the present study and [12]). The lower development from unsynchronized cell populations may have been a consequence of inappropriate cell cycle coordination in approximately 40% of reconstructed embryos, as they would have received cells that were not in G1 [10, 18], and/or a consequence of inadequate nuclear reprogramming from using nonquiescent cells. It is significant to note, however, that the proportion of embryos transferred that resulted in viable calves was not different (around 10%) between MII cytoplasts reconstructed with donor cells presumed to either be in G0 ([12] and this study) and in G1 [10]. Further investigations are needed: first to verify the exact stages of the cell cycle being used in nuclear transfer studies, and then to determine the effects of various cell cycle combinations between cultured somatic cells and cytoplasts on subsequent in vitro and in vivo development.

For the FBA treatment protocol, it is important to ensure that the cytoplasts were not prematurely activated, particularly during electro-fusion. Under the experimental conditions used here, and in agreement with Stice and colleagues [18], a negligible proportion of reconstructed embryos in the FBA group would have been activated (around 1%) by the electrical stimulation employed to achieve fusion at 22–24 hpm,. Despite the presence of calcium and magnesium in the fusion buffer, added to increase fusion rates [12, 13], the young age of the cytoplasts in combination with the electrical fusion parameters used did not result in premature activation of the reconstructed embryos before exposure to ionomycin and 6-DMAP at 30 hpm.

With the introduction of the donor nucleus before activation, it is vital to control the ploidy of the reconstructed embryo after the activating stimulus is applied, in order for normal development to proceed. The relatively high rates of embryo development in the FBA group here may therefore have been in part due to the presence of 6-DMAP in the medium following exposure to ionomycin. This protein kinase inhibitor may have inhibited phosphorylations necessary for the spindle apparatus (as suggested in [36]) and therefore prevented micronuclei formation known to occur when fusion precedes activation [11]. Other researchers have used nocodazole as a microtubule inhibitor to control ploidy, but in their studies in sheep, with a quiescent embryonic cell line [1], there was no apparent benefit of FBA treatment, either in terms of embryo development or embryo survival. The lack of any treatment to prevent micronuclei formation may partly explain the poor embryo and fetal development in an earlier cloning study [29] following the transfer of a small number of embryos derived from bovine cumulus cells, presumably in G0/G1 [4] and apparently exposed to MII cytoplasm for a short period before electrical activation. In the mouse, a polar body is typically extruded after activation of MII cytoplasts reconstructed with donor nuclei [37, 38], quite unlike the situation with sheep and cattle [15]. Cytochalasin B was therefore added to control ploidy when mouse cumulus cells were fused before activation in embryos reconstructed by nuclear transfer [3].

This study also examined the recloning of embryos initially produced after nuclear transfer with quiescent granulosa cells. The aim was to investigate the effect of additional exposure of transferred nuclei to oocyte cytoplasmic factors, in order to allow a longer opportunity for nuclear reprogramming to occur by effectively passing the original differentiated nucleus through two rounds of early embryo development. In amphibians, recloning improved the developmental capacity of terminally differentiated nuclei, resulting in more advanced larval development compared to a single round of nuclear transfer [9]. However, in the experiments here, there was no improvement in terms of either embryo or fetal development compared to first-generation FBA cloned embryos derived directly from granulosa cells. The activation protocol in both the FBA and recloning groups was the same and involved a combination of ionomycin and 6-DMAP, although cytoplasts were activated at different ages: 30 and 24 hpm, respectively. Despite the difference in timing, the efficiency of oocyte activation in control oocytes, as evidenced by pronuclear formation, was the same (both 95%) and similar to that in a previous report [36]. In the experiments here, the blastomeres used for recloning were obtained from compacting morulae in excess of 30 cells. At this stage of development, the transcription of embryonic genes may have commenced in the first-generation cloned embryo [39]. It may be beneficial to reclone embryos before the expected onset of transcription. However, it is difficult at the 8-cell stage to visually identify those cloned embryos that are likely to have the potential for further development. Thus, compacting morulae were recloned, as our experience shows that good-quality morulae reliably develop into good-quality blastocysts. In the experiments here, preactivated (presumptive S-phase) cytoplasts, capable of accepting nuclei at any stage of the cell cycle [25], were fused with unsynchronized blastomeres. It may be necessary to investigate alternative cell cycle coordination options to improve developmental rates.

It is widely acknowledged that nuclear transfer, even with embryonic blastomeres, results in increased rates of abortion throughout pregnancy, high birth weight, perinatal deaths, and poor adaptation to extra-uterine life [40–42]. These effects appear more extreme with somatic cell nuclear transfer [1, 2, 10, 31, 32, 43] and may relate to one deficiency or a combination of deficiencies in either the nuclear transfer procedure itself, leading to incomplete nuclear reprogramming of the cultured donor cells, or in the in vitro maturation and embryo culture systems used. These deficiencies, either collectively or singularly, may lead to inappropriate patterns of gene expression at specific key stages during embryo, fetal, or placental development, contributing to pregnancy loss.

The survival of adult cloned embryos reconstructed in the FBA treatment 60 days after transfer, as indicated by ultrasonography, was relatively high (45%) in this study. This is similar to data from our laboratory with twin embryo transfer of bovine embryos produced after either nuclear transfer with quiescent fetal cells [12] or IVP [44], and is higher than with cloned embryos derived from either nonquiescent fetal cells [10] or embryonic blastomeres [33, 34]. However, the nuclear transfer process in cattle with both cultured embryonic [18] and fetal cells [12] and embryonic blastomeres [33, 34] is currently associated with

high rates of fetal loss throughout gestation. This was exemplified here, with 78% of adult cloned fetuses present at Day 60 not surviving to term, compared to a typical loss of 30% with bovine IVP embryos [23, 45]. It appears that the failure of normal placentation is a problem frequently observed with cloned embryos and also with a proportion of IVP embryos. Approximately 25% of the early embryonic mortality with the IVP embryo appears to be due to an unsuccessful transition from yolk sac to allantoic nutrition, whereby the growth of the allantois is severely retarded, or even nonexistent, and is characterized by a lack of vascularization by Day 34 [46]. It is therefore likely that the IVP cloned embryo will have a similar deficiency during this stage of development. There are reports of high fetal losses during the middle of the first trimester [12, 18]. Despite apparently normal fetal development, part of this loss may be due to a failure of normal placentome development [18]. These losses coincide with the stage at which functioning placentomes are required for the exchange of nutrients and gases [47], and this may in part be due to a deficiency in the underlying allantois (A.J. Peterson, personal communication). The majority of the fetal losses in the third trimester in this study were a consequence of hydrallantois in three twin-bearing recipient cows. This excessive accumulation of allantoic fluid may be a consequence of low numbers of placentomes, leading to placental dysfunction [48]. Reports in the literature with both IVP and cloned conceptuses describe increased incidence of hydrallantois in late gestation, fewer and enlarged placentomes, enlarged umbilical vessels, and edematous placental membranes [10, 42, 49, 50]. These abnormalities of placentation were all observed in some (but not all) recipients here. It is suggested that the embryo survival rate may have been greater had embryos in this study been transferred singularly to minimize pregnancy complications.

With the nuclear transfer pregnancies reported here, it appeared that the appropriate signaling in preparation for birth did not occur normally and there was a lack of "communication" between the maturing fetus and the recipient cow in the weeks leading up to expected full term (Day 282). This was characterized by few overt signs of readiness for birth and by inadequate mammary gland development in the recipient cows over this time. This may reflect another abnormality in placental function, as there are both direct and indirect actions of placental hormones on mammogenesis during pregnancy [51]. The deficiencies in parturition and mammogenesis noted here have been experienced by us previously with sheep [31, 32], and have prompted the current decision to deliver cloned offspring by elective cesarean section. Because of the decision to deliver calves between Days 276 and 281, and to not allow a sufficiently long period for them to calve following corticosteroid injection [52], we did not provide the opportunity to observe whether parturition would have been initiated after induction, or whether it might have occurred naturally at some later point in gestation. Offspring generated from both IVP [50, 53, 54] and cloned embryos [40, 42] do tend to have longer gestation lengths. If, however, there is a problem with either the fetal hypothalamic-anterior pituitary-adrenal axis and/or the transduction of the resulting rise in fetal cortisol near the time of birth to the cow [55], it certainly has not compromised the viability of the cloned calves themselves. Normally, the transmission of fetal cortisol to the dam is mediated by the changing activities of steroidogenic enzymes in the placenta [56], leading to an elevation in maternal estrogen, which in turn initiates the cascade of events leading to parturition [55]. Currently, it is not known whether the cloned calves had naturally high levels of cortisol, as the recipient cows each received an injection of corticosteroid 17 h before cesarean section in order to hasten fetal lung maturation [57]. Nevertheless, it is tempting to speculate that the lack of a typical parturition response may have been primarily due to the inability of the placenta to convert progesterone to estrogen.

The embryo culture system used here has no effect on the birth weight of IVP calves [23]. Despite the lack of control data, the birth weights observed here for the singles, but not the twins, tended to be greater than those generally reported in New Zealand for Friesians [58] and following IVP [23]. Although the birth weight of two cloned calves approached 50 kg, these were not as extreme as some reported elsewhere [40, 42]. All but one of the calves delivered were fundamentally viable and only required basic neonatal veterinary assistance. In the other case, the calf did require treatment to alleviate a slow, irregular heartbeat and respiration rate immediately after delivery. The immediate postnatal behavior of the cloned calves was more vigorous than previously reported [41], and all were standing within 30 min to 2 h and had suckled colostrum. It has been suggested that cloned calves exhibit defects in energy metabolism as evidenced by cases of hypothermia, hypoxemia, hypoglycemia, and metabolic acidosis, and that this may be a consequence of abnormal placental function [41]. With the calves reported here, oxygen therapy was provided by us as a standard procedure, and there was evidence of a metabolic imbalance immediately after birth in two of the calves, which was corrected without intervention.

While microsatellite DNA analyses are consistent with the cloned calves' being genetically identical to the Friesian donor cow (Fig. 2), there are differences in the black and white coat color patterns both between the calves and compared to the cow (Fig. 1, c and d). However, the variation is no different from that commonly observed in genetically identical twins that occur either naturally or after embryo bisection (Wells, personal observations). This aspect of piebald patterning appears not to be under absolute genetic control: environmental influences in utero result in a degree of variability in the multiplication and migration of melanoblasts, which form the melanocytes necessary for pigment production in the developing skin of the fetus [59, 60]. Subtle differences in the detail of the pigmentation markings between genetically identical cloned amphibians have also been noted [30].

The development of an efficient method for producing animals from cultured somatic cells after nuclear transfer will have a number of advantages for both agriculture and biomedicine [61, 62]. These particularly relate to new opportunities for introducing precise genetic modifications into livestock species, following homologous recombination in the cultured cells. We have identified a cell population in the adult female that has proven to be relatively successful in generating cloned offspring after nuclear transfer. Although mural granulosa cells do have a specialized function, they are not terminally differentiated, and, when combined with nuclear transfer methods whereby quiescent cells are fused before activation, relatively high rates of embryo development and survival have resulted. Overall, the technology is currently one-quarter the efficiency of IVP in the bovine species. To better enable the commercial application of this technology, however, the efficiency of the procedures needs to be substantially improved. Re-

search is needed to better understand the reprogramming of differentiated nuclei, in order to improve both the establishment and maintenance of healthy conceptuses. Studies should also focus on the development and function of the placenta and on parturition.

ACKNOWLEDGMENTS

We would like to thank John Lange, Andria Miller, Jan Oliver, Martin Berg, Jacqui Forsyth, Katie Cockram, Tony Day, Jeff Grimmett, Peter Davidson, Adam Hitman, and Cathy Evans-Miller for providing assistance with stock husbandry and neonatal veterinary care; Catherine Cameron for data analyses; and Allan Crawford and Dianne Hyndman for microsatellite analyses.

REFERENCES

1. Campbell KHS, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380:64–66.
2. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810–813.
3. Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369–374.
4. Schuetz AW, Whittingham DG, Snowden R. Alterations in the cell cycle of mouse cumulus granulosa cells during expansion and mucification in vivo and in vitro. Reprod Fertil Dev 1996; 8:935–943.
5. Whitfield JF, Boynton AL, Rixon RH, Youdale T. The control of cell proliferation by calcium, Ca^{2+}-calmodulin, and cyclic AMP. In: Boynton AL, Leffert HL (eds.), Control of Animal Cell Proliferation, vol. 1. London: Academic Press; 1985: 331–365.
6. Gurdon JB, Laskey RA, Reeves OR. The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. J Embryol Exp Morphol 1975; 34:93–112.
7. McAvoy JW, Dixon KE, Marshall JA. Effects of differences in mitotic activity, stage of cell cycle, and degree of specialization of donor cells on nuclear transplantation in *Xenopus laevis*. Dev Biol 1975; 45:330–339.
8. Hoffner Orr N, DiBerardino MA, McKinnell RG. The genome of frog erythrocytes displays centuplicate replications. Proc Natl Acad Sci USA 1986; 83:1369–1373.
9. DiBerardino MA, Hoffner NJ, Etkin LD. Activation of dormant genes in specialized cells. Science 1984; 224:946–952.
10. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998; 280:1256–1258.
11. Czolowska R, Modlinski JA, Tarkowski AK. Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. J Cell Sci 1984; 69:19–34.
12. Wells DN, Misica PM, McMillan WH, Tervit HR. Production of cloned bovine fetuses following nuclear transfer with cells from a fetal fibroblast cell line. Theriogenology 1998; 49:330.
13. Wells DN, Misica PM, Forsyth JT, Berg MC, Lange JM, Tervit HR, Vivanco WH. The use of adult somatic cell nuclear transfer to preserve the last surviving cow of the Enderby Island cattle breed. Theriogenology 1999; 51:217.
14. Collas P, Pinto-Correia C, Ponce de Leon FA, Robl JM. Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. Biol Reprod 1992; 46:501–511.
15. Campbell KHS, Loi P, Otaegui PJ, Wilmut I. Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod 1996; 1:40–46.
16. Collas P, Balise JJ, Robl JM. Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. Biol Reprod 1992; 46:492–500.
17. Kwon OY, Kono T. Production of identical sextuplet mice by transferring metaphase nuclei from four-cell embryos. Proc Natl Acad Sci USA 1996; 93:13010–13013.
18. Stice SL, Strelchenko NS, Keefer CL, Matthews L. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. Biol Reprod 1996; 54:100–110.
19. Pieterse MC, Kappen KA, Kruip ThAM, Taverne MAM. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology 1988; 30:751–762.
20. de Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. Mol Reprod Dev 1995; 42:432–436.
21. Thompson JGE, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. The effect of oxygen concentration on the in vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil 1990; 89: 573–578.
22. Gardner DK, Lane M, Spitzer A, Batt P. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins and culturing embryos in groups stimulate development. Biol Reprod 1994; 50:390–400.
23. Thompson JG, Allen NW, McGowan LT, Bell ACS, Lambert MG, Tervit HR. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology 1998; 49:1239–1249.
24. Hagemann LJ, Weilert LL, Beaumont SE, Tervit HR. Development of bovine embryos in single in vitro production (sIVP) systems. Mol Reprod Dev 1998; 51:143–147.
25. Campbell KHS, Ritchie WA, Wilmut I. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod 1993; 49:933–942.
26. Montgomery GW, Sise JA. Extraction of DNA from sheep white blood cells. N Z J Agric Res 1990; 33:437–441.
27. Montgomery GW, Crawford AM, Penty JM, Dodds KG, Ede AJ, Henry HM, Pierson CA, Lord EA, Galloway SM, Schmack AE, Sise JA, Swarbrick PA, Hanrahan V, Buchanan FC, Hill DF. The ovine Booroola fecundity gene (FecB) is linked to markers from a region of human chromosome 4q. Nat Genet 1993; 4:410–414.
28. Eppig JJ, Wigglesworth K, Pendola F, Hirao Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol Reprod 1997; 56:976–984.
29. Collas P, Barnes FL. Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. Mol Reprod Dev 1994; 38: 264–267.
30. Gurdon JB. Nuclear transplantation in eggs and oocytes. J Cell Sci Suppl 1986; 4:287–318.
31. Wells DN, Misica PM, Day AM, Tervit HR. Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts. Biol Reprod 1997; 57:385–393.
32. Wells DN, Misica PM, Day AM, Peterson AJ, Tervit HR. Cloning sheep from cultured embryonic cells. Reprod Fertil Dev 1999; (in press).
33. Stice SL, Keefer CL. Multiple generation bovine embryo cloning. Biol Reprod 1993; 48:715–719.
34. Peura TT, Lewis IM, Trounson AO. The effect of recipient oocyte volume on nuclear transfer in cattle. Mol Reprod Dev 1998; 50:185–191.
35. Ware CB, Barnes FL, Maili-Laurila M, First NL. Age dependence of bovine oocyte activation. Gamete Res 1989; 22:265–275.
36. Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL. Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev Biol 1994; 166:729–739.
37. Cheong H-T, Takahashi Y, Kanagawa H. Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. Biol Reprod 1993; 48:958–963.
38. Kono T, Ogawa M, Nakahara T. Thymocyte transfer to enucleated oocytes in the mouse. J Reprod Dev 1994; 39:301–307.
39. Smith SD, Soloy E, Kanka J, Holm P, Callesen H. Influence of recipient cytoplasm cell stage on transcription in bovine nucleus transfer embryos. Mol Reprod Dev 1996; 45:444–450.
40. Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME, McCalla DF. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. Anim Reprod Sci 1995; 38:73–83.
41. Garry FB, Adams R, McCann JP, Odde KG. Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 1996; 45:141–152.
42. Kruip ThAM, den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 1997; 47:43–52.
43. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KHS. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 1997; 278:2130–2133.

44. McMillan WH, Peterson AJ, Hall DRH, Donnison MJ. Embryo and recipient contributions to embryo loss to day 60 in heifers receiving either one or two in vitro-produced embryos. Theriogenology 1997; 47:370.
45. McMillan WH, Peterson AJ, Hall DRH, Donnison M, Pugh PA, Lambert MG. Is foetal loss random during pregnancy in cattle? In: The 13th International Congress on Animal Reproduction, vol. 2; 1996; Sydney, Australia. Abstract P11–5.
46. Peterson AJ, McMillan WH. Variation in the rate of emergence of the bovine allantois. In: Proceedings of the 29th Annual Conference of the Australian Society for Reproductive Biology; 1998; Perth, Australia. Abstract 23.
47. Melton AA, Berry RO, Butler OD. The interval between the time of ovulation and attachment of the bovine embryo. J Anim Sci 1951; 10: 993–1005.
48. Arthur GH, Noakes DE, Pearson H. Veterinary Reproduction and Obstetrics (Theriogenology), 6th ed. London: Baillière Tindall; 1989: 49–160.
49. Hassler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995; 43:141–152.
50. van Wagtendonk-de Leeuw AM, Aerts BJG, den Daas JHG. Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. Theriogenology 1998; 49:883–894.
51. Mepham TB. Physiology of Lactation. Milton Keynes: Open University Press; 1987: 109–128.
52. MacDiarmid SC. Induction of parturition in cattle using corticosteroids: a review. Part 1. Reasons for induction, mechanisms of induction, and preparations used. Anim Breed Abstr 1983; 51:403–419.
53. Walker SK, Heard TM, Bee CA, Frensham AB, Warnes DM, Seamark, RF. Culture of embryos of farm animals. In: Lauria A, Gandolfi F (eds.). Embryonic Development and Manipulation in Animal Production: Trends in Research and Applications. London: Portland Press; 1992: 77–92.
54. Numabe T, Oikawa T, Satoh H, Takada N, Horiuchi T. Birthweights of calves conceived by transfer of Japanese black cow embryos produced in vitro. Theriogenology 1998; 47:378.
55. Nathanielsz PW. The timing of birth. Am Sci 1996; 84:562–569.
56. Flint APF, Ricketts AP, Craig VA. The control of placental steroid synthesis at parturition in domestic animals. Anim Reprod Sci 1979; 2:239–251.
57. Rebello CM, Ikegami M, Ervin MG, Polk DH, Job AH. Postnatal lung function and protein permeability after fetal or maternal corticosteroids in preterm lambs. J Appl Physiol 1997; 83:213–218.
58. Baker RL, Carter AH, Morris CA, Johnson DL. Evaluation of eleven cattle breeds for crossbred beef production: performance of progeny up to 13 months of age. Anim Sci 1990; 50:63–77.
59. Silvers WK. The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction. New York: Springer-Verlag; 1979: 185–205.
60. Olsen TA. The genetic basis for piebald patterns in cattle. J Hered 1981; 72:113–116.
61. Baker RL, Shannon P, Garrick DJ, Blair HT, Wickham BW. The future impact of new opportunities in reproductive physiology and molecular biology on genetic improvement programmes. Proc N Z Soc Anim Prod 1990; 50:197–210.
62. Wells DN, Misica PM, Tervit HR. Future opportunities in livestock production and biomedicine from advances in animal cloning. Proc N Z Soc Anim Prod 1998; 58:32–35.

PATENT
(700/202D)
Attorney Docket No. 112800.401

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Keith Henry Stockman Campbell et al.	)	Group Art Unit: 1632
	)	
Serial No.: 09/225,233	)	Examiner: D. Crouch
	)	
Filed: January 4, 1999	)	
	)	

For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER

Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION OF DR. DAVID WELLS UNDER 37 C.F.R. § 1.132

I, David Wells, declare that:

1. Since 1992, I have held the position of Research Scientist, AgResearch, Ruakura, New Zealand. I am a principal researcher in the field of nuclear transfer with embryonic and somatic cells and have been involved in the establishment of embryonic stem (ES) cell technology at AgResearch. In part, my research involves cloning livestock animals, particularly sheep and cattle, from cultured cells using nuclear transfer into oocytes. I have conducted many studies using nuclear transfer into oocytes and developing the resultant embryos into fetuses and animals. A copy of my Curriculum Vitae is attached hereto.

2. From 1987 to 1988, I held the position of Research Scientist, MAF Technology, Ruakura, New Zealand. During my employment with MAF Technology I developed an embryo splitting technique, which was integrated into a large scale

multiple ovulation and embryo transfer program conducted at Hopu Hopu Quarantine Research Station in New Zealand. My research involved cloning sheep by embryo splitting. This involved embryo manipulation and culture and the development of identical twin animals by transfer into hosts.

3. In 1991, I graduated with a Doctor of Philosophy from the University of Edinburgh, UK. I conducted the research for my PhD thesis at the Department of Reproduction and Development, AFRC Institute of Animal Physiology and Genetics Research, Roslin Institute, and the Department of Genetics, at the University of Edinburgh. During my PhD program, I worked in Dr. Ian Wilmut's laboratory and developed competence in embryonic stem (ES) cell isolation. My work involved using isolated ES cells to generate germline chimeras by embryo manipulation and culture and the development of animals.

4. I have published numerous manuscripts in the area of cloning livestock animals from cultured cells using nuclear transfer. Two representative manuscripts are: "Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts" published in *Biology of Reproduction* 57: 385-393 (1997) and "Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells" published in *Biology of Reproduction* 60: 996-1005 (1999).

5. I have read an article by McLaughlin et al. in *Reproduction Fertil. Develop.* 2, 619-622 (1990), a copy of which is attached hereto.

6. McLaughlin et al. discusses the production of embryos and lambs by nuclear transfer using sheep embryonic cells as nuclear donors.

A1

7. Based on McLaughlin et al. and on my experience in cloning mammals, the embryos and sheep of McLaughlin et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and sheep of McLaughlin et al. were generated from sheep embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and sheep of McLaughlin et al. had two parents, one male and one female. The embryos and sheep of McLaughlin et al. had a chromosomal donation from each of these parents. Therefore, embryos and sheep of McLaughlin et al. do not contain the same set of chromosomes as either of their parents.

8. Based on McLaughlin et al. and on my experience in cloning mammals, the embryos and sheep of McLaughlin et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

9. I have read an article by Prather et al. in *Biology of Reproduction* 41, 414-418 (1989), a copy of which is attached hereto.

10. Prather et al. discusses the production of embryos and pigs by nuclear transfer using pig embryonic cells as nuclear donors.

11. Based on Prather et al. and on my experience in cloning mammals, the embryos and pigs of Prather et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and pigs of Prather et al. were generated from pig embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and pigs of Prather et al. had two parents, one male and one female. The embryos and pigs of

Prather et al. had a chromosomal donation from each of these parents. Therefore, embryos and pigs of Prather et al. do not contain the same set of chromosomes as either of their parents.

12. Based on Prather et al. and on my experience in cloning mammals, the embryos and pigs of Prather et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

13. I have read an article by Yong et al. in *Theriogenology* 35, 299 (1991), a copy of which is attached hereto.

14. Yong et al. discusses the production of embryos and goats by nuclear transfer using goat embryonic cells as nuclear donors.

15. Based on Yong et al. and on my experience in cloning mammals, the embryos and goats of Yong et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and goats of Yong et al. were generated from goat embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and goats of Yong et al. had two parents, one male and one female. The embryos and goats of Yong et al. had a chromosomal donation from each of these parents. Therefore, embryos and goats of Yong et al. do not contain the same set of chromosomes as either of their parents.

16. Based on Yong et al. and on my experience in cloning mammals, the embryos and goats of Yong et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various

techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

17. I have read an article by Cheong et al. in *Biology Reproduction* 48, 958-963 (1993), a copy of which is attached hereto.

18. Cheong et al. discusses the production of embryos and mice by nuclear transfer using mouse embryonic cells as nuclear donors.

19. Based on Cheong et al. and on my experience in cloning mammals, the embryos and mice of Cheong et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and mice of Cheong et al. were generated from mouse embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and mice of Cheong et al. had two parents, one male and one female. The embryos and mice of Cheong et al. had a chromosomal donation from each of these parents. Therefore, embryos and mice of Cheong et al. do not contain the same set of chromosomes as either of their parents.

20. Based on Cheong et al. and on my experience in cloning mammals, the embryos and mice of Cheong et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

21. I have read an article by Yang et al. in *Biology Reproduction* 47, 636-643 (1992), a copy of which is attached hereto.

22. Yang et al. discusses the production of embryos and rabbits by nuclear transfer using rabbit embryonic cells as nuclear donors.

23. Based on Yang et al. and on my experience in cloning mammals, the embryos and rabbits of Yang et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and rabbits of Yang et al. were generated from rabbit embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and rabbits of Yang et al. had two parents, one male and one female. The embryos and rabbits of Yang et al. had a chromosomal donation from each of these parents. Therefore, embryos and rabbits of Yang et al. do not contain the same set of chromosomes as either of their parents.

24. Based on Yang et al. and on my experience in cloning mammals, the embryos and rabbits of Yang et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

25. I have read an article by Sims et al. in *Proc. Natl. Acad. Sci.* 90, 6143-6147 (1993), a copy of which is attached hereto.

26. Sims et al. discusses the production of embryos and bovines by nuclear transfer using bovine embryonic cells as nuclear donors.

27. Based on Sims et al. and on my experience in cloning mammals, the embryos and bovines of Sims et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and bovines of Sims et al. were generated from bovine embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and bovines of Sims et al. had two parents, one male and one female. The embryos

and bovines of Sims et al. had a chromosomal donation from each of these parents. Therefore, embryos and bovines of Sims et al. do not contain the same set of chromosomes as either of their parents.

28. Based on Sims et al. and on my experience in cloning mammals, the embryos and bovines of Sims et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

29. I have read WO 95/17500 published June 29, 1995, of Stice et al., a copy of which is attached hereto.

30. Stice et al. discusses the production of genetically modified embryos and mammals by nuclear transfer using genetically modified embryonic cells as nuclear donors.

31. Based on Stice et al. and on my experience in cloning mammals, the genetically modified embryos and mammals of Stice et al. did not receive their chromosomes from a single parental mammal. This is because the genetically modified embryos and mammals of Stice et al. were generated from genetically modified embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the genetically modified embryos and mammals of Stice et al. had two parents, one male and one female. The genetically modified embryos and mammals of Stice et al. had a chromosomal donation from each of these parents. Therefore, the genetically modified embryos and mammals of Stice et al. did not receive their chromosomes exclusively from one of their parents.

32. Based on Stice et al. and on my experience in cloning mammals, the genetically modified embryos and mammals of Stice et al. could be distinguished from an embryo or mammal that receives its chromosomes from a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

33. Based on my experience with mammals cloned by nuclear transfer and mammals propagated by sexual reproduction, the source of a mammal's chromosomes can be readily determined using genetic analysis. By using genetic analysis, whether a mammal is cloned asexually by somatic cell nuclear transfer or propagated by sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, can be determined by comparing the chromosomal DNA of the mammal to that of its parent(s). Only a mammal cloned by somatic cell nuclear transfer will contain the same set of chromosomes as a single parental mammal.

34. Based on my experience with mammals cloned by nuclear transfer and mammals propagated by sexual reproduction, a mammal cloned by somatic cell nuclear transfer is unlike any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell. The set of chromosomes of a mammal cloned by somatic cell nuclear transfer is obtained from a single parental mammal. The set of chromosomes from any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, comes from two parental mammals, one male and one female. This feature allows the cloned mammal to preserve the genetic information of the parental mammal without dilution.

35. Based on my experience with cloned mammals, a mammal that contains the same set of chromosomes as a single parental mammal can be distinguished from the parental mammal due to environmental influences. First, the cloned mammal will always be of a younger age than the parental mammal. Second, the cloned mammal will have a variety of phenotypic differences from the parental mammal, for example, differences in fur and skin pigmentation. Third, the cloned mammal will have behavioral differences from the parental mammal.

36. The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Dated: 5th February 2003

By: D. W. Wells
David Wells, Ph.D

Related Proceedings Appendix to Appeal Brief Under Rule 41.37(c)(1)(x)

None. Although an appeal was filed in U.S. application 09/658,862, there is no decision yet in that case.

The Board's decisions in Interference Nos. 104,746 and 105,192 are attached.

There are no decisions yet in Case Nos. 1:05-cv-00353 RMU and 1:05-cv-00706-RMU.

The opinion in support of the decision being entered today is not binding precedent of the Board.

Paper 123

Filed by: Trial Section Merits Panel
Mail Stop Interference
P.O. Box 1450
Alexandria, VA 22313-1450
Tel: 571-272-9797
Fax: 571-273-0042

Filed
20 December 2004

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES
(Administrative Patent Judge Nagumo)

STEVEN L. **STICE**,
JOSE CIBELLI, JAMES ROBL, PAUL GOLUEKE,
F. ABEL PONCE de LEON,
and D. JOSEPH JERRY,

Junior Party,
(Patent 5,945,577),

v.

KEITH HENRY STOCKMAN **CAMPBELL**
and IAN WILMUT,

Senior Party,
(Application 09/650,194).

Patent Interference No. **104,746**

Before: McKELVEY, Senior Administrative Patent Judge, LANE, and NAGUMO, Administrative Patent Judges.

NAGUMO, Administrative Patent Judge.

DECISION ON PRIORITY

I. Introduction

This interference relates to methods for cloning certain large farm animals, namely cattle, sheep, and pigs, by transferring the nucleus of a differentiated cell (a fibroblast) into a prepared oocyte at a specified stage of development. A merits panel held that junior party Stice was not entitled to a patent on any of its involved claims, which were claims 1-24 of its U.S. patent No. 5,945,577. (Paper 80 at 37-40.) The interference was redeclared with three counts, Counts 4 through 6, based solely on certain surviving claims of senior party Campbell. (Paper 81 at 3.)

An oral hearing on priority was held in the presence of a court reporter on 15 November 2005. (See Paper 120, transcript of oral argument.) Ronald A. Daignault, Esq., argued for Stice. Kenneth J. Meyers, Esq., accompanied by David J. Earp, Esq., argued for Campbell.

II. Findings of fact

The record supports the following findings of fact as well as any other findings of fact set forth in any other portion of the decision by at least a preponderance of the evidence.

The interference

1. This interference was declared on 30 January 2002, between junior party Steven L. Stice, Jose Cibelli, James Robl, Paul Golueke, F. Abel Ponce de Leon, and D. Joseph Jerry ("Stice") and senior party Keith Henry Stockman Campbell and Ian Wilmut ("Campbell").

2. Stice is involved in the interference on the basis of its 5,945,577 ("577") patent, issued on 31 August 1999, and based on application 08/781,752, filed 10 January 1997.

3. According to Stice, its real party in interest is "the University of Massachusetts, which has exclusively licensed their interest to Advanced Cell Technology Corporation" (Paper 9).

4. Campbell is involved in the interference on the basis of its 09/650,194 ("194") application, filed 29 August 2000.

5. According to Campbell, its real party in interest is

(1) Assignee: Roslin Institute (Edinburgh) of Midlothian, England;

(2) licensees: Geron Corporation, of Menlo Park, CA, and Exeter Life Sciences, Inc., of Phoenix, AZ. (Paper 98 at 2.)

The counts

6. **Count 4** reads as follows (Paper 81 at 3):

A method according to any of claims 19 or 23 of Campbell application 09/650,194.

7. **Count 5** reads as follows (Paper 81 at 3):

A method according to any of claims 27 or 31 of Campbell application 09/650,194.

8. **Count 6** reads as follows (Paper 81 at 3):

A method according to claim 35, claim 39, claim 43 or claim 47 of Campbell application 09/650,194, where the "non-human mammal" is a pig or a porcine and where the "non-human mammalian fetus" is a pig fetus or a porcine fetus.

9. Claim 23 of Campbell reads as follows:

A method of cloning a bovine fetus by nuclear transfer comprising:

(i) inserting a nucleus of a cultured diploid bovine fibroblast in the G1 phase of the cell cycle into an unactivated, enucleated metaphase II-arrested bovine oocyte to reconstruct an embryo;

(ii) maintaining the reconstructed embryo without activation for a sufficient time to allow the reconstructed embryo to become capable of developing to term;

(iii) activating the resultant reconstructed embryo;

(iv) culturing said activated, reconstructed embryo to blastocyst; and

(v) transferring said cultured, reconstructed embryo to a host cow such that the reconstructed embryo develops into a fetus.

10. The other Campbell claims referred to in the counts are independent claims that are also directed to methods of cloning by nuclear transfer comprising the same overall steps as Campbell claim 23. The following differences are noted:

(a) Campbell claim 19 is directed to a method of cloning a cow.

(b) Campbell claim 27 is directed to a method of cloning a sheep.

(c) Campbell claim 31 is directed to a method of cloning an ovine fetus.

(d) Campbell claim 35 is directed to a method of cloning a non-human mammal.

(e) Campbell claim 39 is directed to a method of cloning a non-human mammalian fetus.

(f) Campbell claim 43 is directed to a method of cloning a non-human mammal and requires that the donor cell be a differentiated cell.

(g) Campbell claim 47 is directed to a method of cloning a non-human mammalian fetus and requires that the donor cell be a differentiated cell.

Claim correspondence

11. The claim correspondence was not disturbed by the redeclaration of this interference. (Paper 83.)

a. The claims corresponding to **Count 4** are:

Stice: 1-24

Campbell: 19-26 and 35-50

b. The claims corresponding to **Count 5** are:

Stice: 1-22

Campbell: 27-50

c. The claims corresponding to **Count 6** are:

Stice: 1-22

Campbell: 35-51[1].

[1] Campbell was authorized to file an amendment adding claim 51 to its involved application (Paper 32 at 2). Campbell claim 51 is directed to a method of cloning a pig using the nuclear transfer method and thus corresponds to Count 6.

12. No Stice claims are patentable. (Paper 80 at 40, 43.)

Benefit

13. Stice was not accorded priority benefit of the filing date of any prior application (Paper 81 at 3).

14. Campbell was accorded priority benefit of the following three applications for all three counts of the interference (Paper 81 at 3):

US application 08/803,165, filed 19 February 1997, and issued as patent 6,252,133 on 26 June 2001;

PCT application PCT/GB96/02098, filed 30 August 1996; and

GB application 9517779.6, filed 31 August 1995.

15. The parties continue to rely on their original priority statements. (Paper 83 (Campbell); Paper 84 (Stice).)

Arguments

16. Stice filed a principal brief on priority (Paper 92), which Campbell opposed (Paper 104); Stice filed a reply (Paper 106).

17. Stice subsequently filed a corrected brief (Paper 115) pursuant to an Order (Paper 112) to renumber its exhibits consecutively.

18. Campbell filed a principal brief on priority (Paper 99), which Stice did not oppose, as Campbell noted in a "Notice concerning filing of reply brief" (Paper 107).

Stice case for priority

Conception

19. Stice points to an entry dated "6/22/95" in a laboratory notebook prepared by Dr. Steven L. Stice as evidence of conception. (Paper 115 at 8.)

20. According to Stice, the critical sentence supporting conception reads:

"Want to try electroporation on fibroblast so that they can be used to produce nuclear transfer embryos from clonal cells. Will talk to Jose [Cibelli] about this."

(Paper 115 at 8, citing SX 2055[2].)

21. Stice represents that Dr. Stice signed this page of his laboratory notebook on 23 June 1995. (Paper 115 at 8.)

22. Stice represents further that this page was "subsequently corroborated" by Mr. Jeffrey Kane on 30 April 1997.

23. Review of Stice exhibit SX 2055 (Stice notebook) confirms the dates cited by Stice.

24. Exhibit 2055 is not labeled by any page numbers.

---

[2] Stice Exhibits are cited as SX 2___; Campbell exhibits are cited as CX 1___.

25. Kane testified that he signed the notebook on the date indicated. (SX 2052 at 1.)

26. Stice argues that "this is the first date [22 June 1995] indicating the conception by Dr. Steven Stice of a method of using differentiated donor cells (i.e. fibroblast cells) as donor cells for subsequent nuclear transfer experiment from which offspring could be generated. The conception corresponds to the subject matter of the Counts." (Paper 115 at 8-9.)

27. Stice has not, in its principal brief, directed our attention to any expert testimony or other evidence supporting its assertions regarding the critical sentence and its relation to any of counts 4-6.

28. Dr. Stice testified, "[t]hat entry [SX 2055] indicates that I wanted to try the process of electroporation on fibroblst cells in order that they may be used to produce nuclear transfer embryos from the cloned cells. I also indicated in my notebook that I wanted to talk to my colleague, Dr. Jose Cibelli, regarding developing this technology." (Stice declaration, SX 2050 at 2-3.)

29. We find no explanation in Stice's declaration of the relation of his sentence to the limitations of any of the Counts.

30. According to Stice, on another page of Dr. Stice's laboratory notebook, in an entry dated 27 June 1995, Dr. Stice

wrote, "Try to use the electroporation to introduce β-geo into fibroblast cells bovine (Jose's [Cibelli]). The idea is to use these transgenic cells in NT [nuclear transfer] to produce fetuses and offspring? machine?" (SX 2056; square-bracketed material added by Stice.)

31. Exhibit 2056 is not labeled by any page numbers.

32. Review of SX 2056 indicates that Dr. Stice wrote "Tried", rather than "Try" in the sentence quoted by Stice.

33. Dr. Stice appears to have signed and dated this page on 28 June 1995. (SX 2056.)

34. Exhibit 2056 also shows a partial signature, "Jeffrey J. Ka", and a partial date, "April 30". (SX 2056.)

35. Kane testified that he reviewed and signed the pages of the laboratory notebooks shown in SX 2055 and SX 2056 in 1997. (SX 2052 at 1, ¶2.)

36. Stice offers, as collaboration of Dr. Stice's conception, the signature and declaration of Mr. Jeffrey Kane (SX 2052). (Paper 115 at 8.)

Reduction to practice

37. Stice provides a "summary" of Cibelli's research notebooks (SX 2057 and 2058) covering the period 10 August 1995 through 30 August 1996. (Paper 115 at 10-21.)

38. Stice urges that it achieved an actual reduction to practice of subject matter within the scope of Count 4 on or about 30 August 1996, as shown by an entry in a notebook maintained by Cibelli. (Paper 115 at 21.)

39. Stice characterizes the actual reduction to practice as one in which "successful nuclear transfer fusions using differentiated fibroblast cells were completed leading to viable offsprings." (Paper 115 at 21.)

40. The summary for activities of 30 August 1996 refers to pregnancies of "7/30/96." (Paper 115 at 20.)

41. There is no entry in the summary table for 30 July 1996.

42. Stice, in its principal brief, has not directed our attention to any expert testimony that explains any of the summaries of Cibelli's notebooks, nor how the summaries relate to any of the counts in this interference.

43. Stice, in its principal brief, has not made any arguments relating the evidence on which it ultimately relies to support its actual reduction to practice, namely Cibelli's notebooks, SX 2057 and SX 2058, to the limitations of any of the counts.

44. Stice, in its principal brief, has not directed our attention to any expert testimony explaining the significance of

any entries in Cibelli's notebooks vis-à-vis any count; nor are any of the data in the notebook explained as to their origin, meaning, or reliability.

Diligence

45. Campbell has been accorded the benefit for priority in this interference of its UK application, filed on 31 August 1995. (Paper 81 at 3)

46. Stice cites a second entry from Dr. Stice's notebook as evidence of the beginnings of a "concerted effort to reduce this invention to practice: "Try [sic: Tried] to use the electroporation to introduce β-geo into fibroblast cells bovine (Jose's). The idea is to use these transgenic cells in NT to produce fetuses and offspring? machine?"). (Paper 92 at 10, square bracketed remarks added by Stice omitted.)

47. Stice provides a table that begins at page 10 of its priority brief and runs to the top of page 21, in which dates are paired against brief descriptions of activity, which are said to be summaries of notebook pages from Dr. Jose Cibelli's laboratory notebook.

48. Stice, in its principal brief, has not directed our attention to any testimony explaining the meaning or significance of any entries in the table.

49. The entries in the table are dated from 10 August 1995 through 30 August 1996.

50. Stice's table starts 22 days before Campbell's priority benefit date of 31 August 1995.

51. Stice's table indicates activity on 10 of the 22 days before 31 August 1995.

52. The 12 days of inactivity between the start of the Stice Table and 31 August 1995 comprise four blocks of three days each.

53. Stice has not offered any explanation of these periods of silence.

54. Of the 365 days covered by Stice's table following 31 August 1995, 149 are days for which some activity was reported. and 216 are days without reports of activity.

55. From a calendar marked up to indicated the days of activity chronicled in Stice's table, it is apparent that there are several larger blocks of time after Campbell's priority benefit date during which no activity was reported.

a. The largest block of unexplained inactivity extends 18 days, from 29 June 1996 through 16 July 1996.

b. The second largest block of unexplained inactivity covers 16 days, from 4 November 1995 through 19 November 1995.

c. The remaining periods of unexplained inactivity after 31 August 1995 are all of shorter duration – there are three seven-day periods, three six-day periods, and six five-day periods of unexplained inactivity; we have not counted the number of shorter periods of inactivity following 31 August 1995.

d. The sum of these shorter periods of inactivity is 223 days.

56. Stice, in its principal brief, has neither identified nor explained any gaps of activity during the period covered by the table.

57. Stice, in its principal brief, has not described any activities from the period 30 August 1996 through 10 January 1997, the filing date of the application that resulted in its involved patent.

a. This last period of unexplained inactivity covers 133 days.

58. Stice offers, as corroboration of its actual reduction to practice and diligence, the signature of Ms. Catherine Blackwell ("Blackwell") on Cibelli's notebook pages presented in SX 2057, and her declaration.

59. Blackwell declares, "[b]eginning during the period of approximately June 1995, I was aware of a research project conducted primarily by Drs. Stice and Cibelli regarding the issue

of differentiated cells in nuclear transfer technology. Their goal was to produce embryos from the cloned differentiated cells." (SX 2053 at 2, ¶ 2; SX 2054 at 2, ¶2.)

Cibelli's notebooks

60. Dr. Jose B. Cibelli ("Cibelli") states, "[w]hile affiliated with Advanced Cellular Technology, my colleague and I, Dr. Steven Stice, began a series of experiments on or about June 1995 and continuing through the end of 1996, using nuclear transfer techniques with differentiated cells in the hopes of developing a method for produced cloned animals." (SX 2051 at 2, ¶ 3.)

61. Copies of Cibelli's notebook pages are presented in SX 2057 and SX 2058.

62. SX 2058 consists of two parts.

63. Part one of SX 2058 consists of pages 1 (cover) through 59 of a 96-page bound notebook, with dated entries running from "8/10/95" through "2/5/96".

64. Page numbers 2 through 59 of SX 2058 are handwritten entries at the bottom center of each page; the cover of the first notebook is not numbered.

65. Pages 1-59 of SX 2058 are not signed by the writer.

66. Pages 1-59 of SX 2058 are not witnessed, i.e., signed and dated by another, signifying that the pages have been read and understood.

67. Page 59 of SX 2058 has the notations "END OF BOOK", what appears to be signature of Jose Cibelli, and "NEXT BOOK ACT#9".

68. Part two of SX 2058 appears to consist of the cover of "NOTEBOOK NO. 9" and pages 1-80 (so labeled, apparently by the manufacturer) of that notebook.

69. Page 60 of SX 2058 appears to be the cover of "Notebook No. 9," issued to Jose Cibelli, Department ACT, returned 7 August 1997.

70. Page numbers, "60" through "141", have been entered by hand at the bottom center of each page of part two of SX 2058.

71. Pages 60-141 of SX 2058 appear generally to have been signed by Jose Cibelli on the last date noted on the body of each page.

72. Pages 61-141 appear to have been witnessed (signature illegible) in blocks at intervals of a few weeks.

73. The first entry is dated "2/5/96" and witnessed "2-9-96".

74. The last entry is dated "7/26/96" and witnessed "7-30-96".

75. SX 2057 (corrected) covers pages 81 to 102 (pre-numbered), 26 July 1996, through 4 September 1996, of a notebook, which appears to be the continuation of "Notebook No. 9".

76. Pages 81-102 are signed and dated as in SX 2058, "Notebook No. 9".

77. Page 81 is dated "7/26/96". (SX 2057).

78. Page 102 is dated "9/4/96". (SX 2057).

79. Pages 81-102 also bear the witness signature of Catherine E. Blackwell and the witness date of "5/8/97". (SX 2057).

Campbell case for priority

80. Campbell rests its case for priority on its British Application No. GB 95 17779.6 (CX 1003), filed 31 August 1995, the benefit for priority of which it has been accorded. (Campbell Principal Brief on Priority, Paper 99 at 2.)

81. Stice has not filed a brief in opposition to Campbell's case in chief for priority.

III. Discussion

The senior party in an interference is presumed to be the first inventor. 37 CFR § 41.207(a)(1) (2004)[3]. The junior party

[3] New regulations governing interferences before the United States Patent and Trademark Office were published at 69 Fed. Reg. 49,960 (12 August 2004), effective 13 September 2004. Except in those instances in which a party would be

bears the burden of proving, by a preponderance of the evidence, a prima facie case that it was the prior inventor. The party filing a motion has the burden of proof to establish that it is entitled to the requested relief. 37 CFR § 41.121(b) (2004); *Velander v. Garner*, 348 F.3d 1359, 1369-70, 68 USPQ2d 1769, 1777 (Fed. Cir. 2003). As a consequence, if the junior party fails to make out a prima facie case that it was the prior inventor, the senior party is awarded judgment by default.

A party who was not the first to file an application for patent of the interfering invention may nonetheless be adjudged the first inventor if it proves that it was the first to conceive of an embodiment of the interfering invention, and that it worked diligently to reduce an embodiment of the interfering invention to practice from a time before the senior party conceived of its invention until the junior party reduced its invention to practice. "Priority and its constituent issues of conception and reduction to practice are questions of law predicated on subsidiary factual findings." *Eaton v. Evans*, 204 F.3d 1094, 1097, 53 USPQ2d 1696, 1698 (Fed. Cir. 2000).

Conception

Conception is the formation "in the mind of the inventor of a definite and permanent idea of the complete and operative

---

prejudiced due to a reliance on the old rules, the new rules shall be applied.

invention, as it is therefore to be applied in practice." *Kridl v. McCormick*, 105 F.3d 1446, 1449, 41 USPQ2d 1686, 1689 (Fed. Cir. 1997) (citations omitted). Conception must include every feature or limitation of the claimed invention. *Id.* Moreover, "[c]onception must be proved by corroborating evidence . . . a reasonable analysis of all the pertinent evidence to determine whether the inventor's testimony is credible. The tribunal must also keep in mind the purpose of corroboration, which is to prevent fraud, by providing independent confirmation of the inventor's testimony.") *Id.* at 1449-50, 41 USPQ2d at 1689; (citations omitted.) Nonetheless, the sufficiency of corroborative evidence must be judged by the 'rule of reason,' under which the tribunal must consider and analyze all pertinent evidence to determine whether the inventor's testimony is credible. *Id.* As the Federal Circuit has emphasized, "Because conception is a mental act, evidence of conception must ultimately address whether the inventor formed 'the definite and permanent idea of the complete and operative invention' in his or her mind." *In re Jolley*, 308 F.3d 1317, 1325, 64 USPQ2d 1901, 1907 (Fed. Cir. 2002).

In the present case, with respect to count 4, junior party Stice seeks to show that it was the first to conceive and that it acted diligently to reduce its invention to practice from a time

before senior party Campbell conceived of its invention. Stice's arguments fail at virtually every level.

First, Stice has failed to show that its evidence of conception, when read by one skilled in the relevant art, discloses every element of the count. The alternative definition of count 4 provided by Campbell claim 23, which recites a method of cloning a bovine fetus, is the most relevant to Stice's proofs. The limitation to which the parties have devoted the most attention is underscored in the first step, which reads as follows:

> (i) inserting a nucleus of a cultured diploid bovine fibroblast <u>in the G1 phase of the cell cycle</u> into an unactivated, enucleated metaphase II-arrested bovine oocyte to reconstruct an embryo.

The evidence on which Stice relies for its proof of conception is directed to fibroblasts generally: "Want to try electroporation on fibroblast so that they can be used to produce nuclear transfer embryos from clonal cells. Will talk to Jose about this." (SX 2055.) This statement, by itself, does not relate to cattle in particular, as recited in Campbell claim 23. As counsel for Stice conceded at oral argument, "there is no cow there." (Paper 120 at 10, l. 12 (erroneously attributed to Mr. Meyers; Mr. Daignault spoke.)) Count 4 specifies further that

the nucleus to be transferred must be a "cultured diploid bovine fibroblast in the G1 phase of the cell cycle." Stice, in its principal brief, has not directed our attention to any testimony or other evidence indicating that one skilled in the art would have recognized that Stice's sentence teaches or discloses the limitation in count 4 that the transferred nucleus be in the G1 phase of the cell cycle. Similarly, the further limitations that a nucleus be implanted into an unactivated enucleated metaphase II-arrested oocyte, and that the reconstructed embryo be maintained "without activation for a sufficient time to allow the reconstructed embryo to become capable of developing to term," are not apparent from the Stice sentence. Stice has not presented any testimony or other evidence to bridge the gap between the sentence and the subject matter of count 4.

Although Stice relies on Dr. Stice's notebook entry of 27 June 1995, as evidence of diligence, we may consider whether this entry is evidence of conception of an embodiment within the scope of any of the counts. Dr. Stice wrote, "Tried to use the electroporation to introduce β-geo into fibroblast cells bovine (Jose's). The idea is to use these transgenic cells in NT to produce fetuses and offspring? machine?" (Paper 92 at 10.) We have no difficulty accepting that "Jose" refers to co-inventor Cibelli, or that "NT" is an abbreviation for "nuclear transfer."

However, although this passage at least discloses that the nucleus to be transferred is bovine, the other limitations, particularly the G1-phase of the transferred nucleus, are not plainly evident from the text.

Stice, in its principal brief, makes no effort to show that all the limitations required by the count are present in either sentence recorded by Dr. Stice. Nor did Stice, in its principal brief, attempt to show that one skilled in the art would have recognized his statement as a clear idea of an embodiment within the scope of count 4. The significance of data and other documentary exhibits must be explained. See, e.g., 37 CFR § 1.671(f) (2003) ("The significance of documentary and other exhibits identified by a witness in an affidavit or during oral deposition shall be discussed with particularity by a witness"); 37 CFR § 1.608(b) (2003) (similar requirement for discussion of the significance of documents); Standing Order §§ 42, 43 (Paper 2) requiring underlying facts be disclosed that form the basis of expert opinion, and explanations of scientific tests and data). Stice's statement that "[t]he conception corresponds to the subject matter of the Counts" is unsupported by any explanation. When questioned on this point at oral argument, Stice urged that the G1-phase limitation was met inherently by a nucleus taken from a culture of propagating cells. (Paper 120 at 11.) The

board considered and rejected this same general argument from Stice regarding whether the transfer of the nucleus of a "proliferating cell" was inherently met if the nucleus was selected from a proliferating cell culture. (Paper 80 at 37-40, discussion Campbell preliminary motion 3.) As Stice has not come forward with any new evidence or argument, we reject its contention that the missing G1 limitation is inherent.

Moreover, Stice has failed to meet the requirement that inventor testimony regarding conception be corroborated. Kane stated that "I do hereby affirm the research that is reported on those pages [SX 2055 and SX 2056] was performed by the investigators who produced the notebooks. I was given these books to sign in 1997 as signed, even though the work was done earlier." (SX 2052 at 1, ¶2.) This statement is insufficient to establish more than that those pages existed on the date Kane signed them. Kane's statement that "the research . . . was performed" begs the questions of exactly what the laboratory notebook entries indicate Dr. Stice conceived, and how that conception, whatever it was, relates to the limitations of the present counts in this interference. Dr. Cibelli's testimony and laboratory notebooks cannot provide corroboration, as Dr. Cibelli is a co-inventor. Unlike the case of *Jolley*, in which inventor McGraw established by "sufficient circumstantial evidence of an

independent nature," 308 F.3d at 1325, 64 USPQ2d at 1907, Stice has presented no probative evidence corroborating either the substance or the date of Dr. Stice's alleged conception.

Accordingly, we hold that Stice has failed to prove conception of an embodiment within the scope of count 4, prior to Campbell's constructive reduction to practice.

Stice has presented no arguments directed to its prior conception of the subject matter of counts 5 and 6, which relate to ovine (sheep) and porcine (pig) embodiments, respectively. All the evidence put forward by Stice that is arguably prior to Campbell's benefit date of 31 August 1995 relates to inventions involving bovines (cattle). Accordingly, we hold that Stice has failed to demonstrate conception of an embodiment of counts 5 or 6 prior to Campbell's constructive reduction to practice.

Actual reduction to practice

Stice's evidence in support of an actual reduction to practice of an embodiment within the scope of count 4 are similarly deficient. Stice made no attempt, in its principal brief, to explain, with the assistance of relevant evidence, including expert testimony, how the activities reported in Cibelli's research notebook on 30 August 1996, amount to a reduction to practice of an embodiment within the scope of

count 4. There is no explanation, by one skilled in the art, of the significance of the entries in Dr. Cibelli's notebooks relating to actual reduction to practice. Even counsel for Stice appear to have been confused about when critical events occurred. Review of the notebook page for "8/30/96" shows no reference to any "pregnancies from 7/30/96," as alleged by Stice. (Paper 115 at 20.) In response to Campbell's Opposition (Paper 104 at 30-31), Stice admitted, "Dr. Cibelli did not always record everything in his notebook, as evidenced by the lack of an entry for July 30, 1996, despite a reference to activities on that date in the August 30, 1996 entry." (Paper 106 at 20.) In Stice's demonstrative exhibit presented at oral argument, however, the summary for August 30, 1996, refers to "the pregnancies of July 25, 1996." (Paper 122 at 4.) When questioned about the discrepancy between Stice's principal brief and its demonstrative exhibit, counsel for Stice stated that the reference to July 25, 1996 in its brief was a typographical error. (Paper 120 at 16.) At another point, counsel for Stice also stated, "I have seen pages where there are three separate experiments, one related to the count and one related probably to a different project." (Paper 120 at 24.)

These statements by counsel emphasize the necessity of testimony by a witness intimately familiar with laboratory

notebooks. Such records are highly technical, and in practice are often rather abbreviated and idiosyncratic documents. The significance of a given entry or series of entries is often not apparent to an outsider, expert or not, although it may become so if explained. Without such testimony – and commentary, if available, from an opposing expert (or perhaps better, an independent expert) – a lay panel cannot reasonably be assured of coming to any reliable conclusions from its own study of the notebooks. In the absence of testimony explaining and evaluating the experimental procedures, tests, and the conclusions that may be drawn from them, we decline to accord any weight to the unexplained raw data of Cibelli's notebooks.

Moreover, there is no independent evidence in the record corroborating Cibelli's experiments or the results. It is well-settled that an inventor seeking to prove an actual reduction to practice "must provide corroborating evidence in addition to his own statements and documents. Such evidence may consist of testimony of a witness, other than an inventor, to the actual reduction to practice or it may consist of evidence of surrounding facts and circumstances independent of information received from the inventor. The purpose of the rule requiring corroboration is to prevent fraud." *Hahn v. Wong*, 892 F.2d 1028, 1033, 13 USPQ2d 1313, 1317 (Fed. Cir. 1989) (internal quotes and

citations omitted). The general testimony of Blackwell and Kane that the inventors were conducting research in nuclear-transfer techniques and animal cloning does not provide surrounding facts and circumstances sufficient to corroborate an actual reduction to practice of an embodiment within the scope of count 4.

Diligence

Stice's case for diligence is similarly flawed. Its summary of activities is mere attorney argument, unsupported by testimony regarding the underlying work reported in Cibelli's laboratory notebooks. Cibelli's broad description of the research program (SX 2051 at 3-4, ¶¶ 6-7) lacks particularity, and does not suffice to explain his notebook entries. Even regarding the summary, there is no explanation of how the activities relate to the limitations of count 4. In the absence of testimony explaining the relation of the activities reported in Cibelli's notebooks to the subject matter of the counts, and in the absence of testimony explaining the gaps in the record, we are unable to assess the significance of Cibelli's notebooks as they relate to diligent efforts to reduce an embodiment of the invention to practice, and we decline to accord them any weight.

Conclusions

As we have stated repeatedly in other cases, we shall not act as an advocate for either of the parties. When arguing a case concerning a rapidly developing, complicated, highly technical art, it is particularly important to explain how the evidence of record relates to the critical legal issue – here, the counts. The opposing party may then admit or deny the validity of the evidence and its relevance to the issues, providing its own evidence, including expert testimony, if appropriate. Following a reply, the tribunal is then in some reasonable position to weigh the merits of the arguments and to determine whether the moving party has carried its burden. Although Stice's Reply Brief, Paper 106, offers considerably more detailed argument than in its Principal Brief, to the extent it attempts to establish a prima facie case of priority, or any of the underpinnings, it is untimely, and we shall not consider them. No good cause has been shown to present new arguments. It is fundamentally unfair to sit back and wait for an opposition, and then attempt to put together a prima facie case in reply, when the opposing party has no opportunity to contest the belated and newly presented arguments.

ORDER

In view of the foregoing considerations, it is:

ORDERED that Stice has failed to establish, by a preponderance of the evidence, that it conceived an embodiment within the scope of any of counts 4-6, which are all the counts of this interference, before Campbell's constructive reduction to practice;

FURTHER ORDERED that Stice has failed to establish, by a preponderance of the evidence, that it reduced to practice an embodiment within the scope of any of counts 4-6;

FURTHER ORDERED that Stice has failed to establish, by a preponderance of the evidence, that it was diligent in its attempts to reduce to practice an embodiment within the scope of any of counts 4-6.

FURTHER ORDERED that Judgment is entered in Paper 124 which accompanies this decision.

FURTHER ORDERED that this paper be given an appropriate number and placed in the patent file of U.S. Patent 5,945,577 and in the application file of 09/650,194.

FURTHER ORDERED that if there is a settlement, the attentions of the parties are directed to 35 U.S.C. § 135(c) and 37 CFR § 41.205.

MCK

FRED E. McKELVEY
Senior Administrative Patent Judge

SALLY GARDNER LANE
Administrative Patent Judge

MARK NAGUMO
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TRIAL SECTION MERITS PANEL

Alexandria, VA
20 December 2004

cc: via first class mail:

Counsel for Stice:

Ronald A. Daignault, Esq.
MERCHANT & GOULD, P.C.
1101 30th Street, N.W.,
Suite 500
Washington, DC 20007

Phone: 202-625-8380
Fax: 202-625-8381

Counsel for Campbell:

Kenneth J. Meyers, Esq.
FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, LLP
1300 I Street, N.W.,
Suite 700
Washington, DC 20005

Phone: 202-408-4000
Fax: 202-408-4400

KJM/SJA
07681.0010-01

The opinion in support of the decision being entered today is not binding precedent of the Board.

Paper 124

Filed by: Trial Section Motions Panel
Mail Stop Interference
P.O. Box 1450
Alexandria, VA 22313-1450
Tel: 571-272-9797
Fax: 571-273-0042

Filed
20 December 2004

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES
(Administrative Patent Judge Nagumo)

STEVEN L. **STICE**,
JOSE CIBELLI, JAMES ROBL, PAUL GOLUEKE,
F. ABEL PONCE de LEON,
and D. JOSEPH JERRY,

Junior Party,
(Patent 5,945,577),

v.

KEITH HENRY STOCKMAN **CAMPBELL**
and IAN WILMUT,

Senior Party,
(Application 09/650,194).

Patent Interference No. **104,746**

Before: McKELVEY, Senior Administrative Patent Judge, LANE, and NAGUMO, Administrative Patent Judges.

NAGUMO, Administrative Patent Judge.

FINAL JUDGMENT – PRIORITY – Bd. R. 127

12.23.04

In view of the discussion Decision on Priority, Paper 123, it is:

ORDERED that adverse judgment as to priority with respect to counts 4, 5, and 6 is entered against Stice;

FURTHER ORDERED that Steven L. Stice, Jose Cibelli, James Robl, Paul Golueke, F. Abel Ponce de Leon, and D. Joseph Jerry are not entitled to a patent containing claims 1-24 of U.S. Patent 5,945,577;

FURTHER ORDERED that this paper be given an appropriate number and placed in the patent file of U.S. Patent 5,945,577 and in the application file of 09/650,194.

FURTHER ORDERED that if there is a settlement, the attentions of the parties are directed to 35 U.S.C. § 135(c) and 37 CFR § 41.205.

FRED E. McKELVEY
Senior Administrative Patent Judge

SALLY GARDNER LANE
Administrative Patent Judge

MARK NAGUMO
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TRIAL SECTION MERITS PANEL

Alexandria, VA
20 December 2004

cc: via first class mail:

Counsel for Stice:

Ronald A. Daignault, Esq.
MERCHANT & GOULD, P.C.
1101 30th Street, N.W.,
Suite 500
Washington, DC 20007

Phone: 202-625-8380
Fax: 202-625-8381

Counsel for Campbell:

Kenneth J. Meyers, Esq.
FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, LLP
1300 I Street, N.W.,
Suite 700
Washington, DC 20005

Phone: 202-408-4000
Fax: 202-408-4400

The opinion in support of the decision being entered today is not binding precedent of the Board.

Paper 93

Filed by: Trial Section Motions Panel
Mail Stop Interference
P.O. Box 1450
Alexandria, VA 22313-1450
Tel: 571-272-9797
Fax: 571-273-0042

Filed
11 February 2005

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES
(Administrative Patent Judge Mark Nagumo)

STEVEN L. **STICE**,
JOSE CIBELLI, JAMES ROBL,
PAUL GOLUEKE, F. ABEL PONCE de LEON
and D. JOSEPH JERRY,

Junior Party,
(Patent 6,235,970),

v.

KEITH HENRY STOCKMAN **CAMPBELL**
and IAN WILMUT,

Senior Party,
(Application 09/989,126).

Patent Interference No. 105,192

Before: MCKELVEY, Senior Administrative Patent Judge, and LANE and NAGUMO, Administrative Patent Judges.

NAGUMO, Administrative Patent Judge.

DECISION - SUBSTANTIVE MOTIONS

I. Introduction

This interference relates to methods for culturing certain Inner Cell Mass cells, which are often referred to in the popular press as "embryonic stem cells." The Inner Cell Mass ("ICM") is the cluster of cells in an embryo at the blastocyst (hollow shell) stage that develops into the fetus. Such cells are called "pleuripotent" because they can differentiate to become any tissue in the developing creature. Researchers hope that ways will be found to grow cultured inner cell mass (CICM) cells into tissues useful for diagnosis and therapy of various conditions arising from injury and disease. The particular CICM cells at issue in this interference arise from the implantation of a prepared nucleus from a differentiated cell into a prepared oocyte (egg cell). The process at the core of this interference involves transferring a prepared nucleus into an enucleated oocyte (an egg cell without its own nucleus). When the embryo reaches the blastocyst stage, several days after fertilization, the inner cell mass cells are harvested and cultured in vitro.

An oral hearing was held in the presence of a court reporter on 15 November 2004. (See Paper 91, transcript of oral argument.) Ronald A. Daignault, Esq., accompanied by Joseph M. Bennet-Paris, Esq., argued for Stice. Kenneth J. Meyers, Esq., accompanied by David J. Earp, Esq., argued for Campbell.

II. Findings of fact

The record supports the following findings of fact as well as any other findings of fact set forth in any other portion of the decision by at least a preponderance of the evidence.

The interference

Background

1. This interference was declared on 30 January 2002, between junior party Steven L. Stice, Jose Cibelli, James Robl, Paul Golueke, F. Abel Ponce de Leon, and D. Joseph Jerry ("Stice") and senior party Keith Henry Stockman Campbell and Ian Wilmut ("Campbell").

2. Stice is involved on the basis of U.S. Patent 6,235,970, issued 22 May 2001 ("970 patent"; SX 2002[1]), which is based on application 08/935,052, filed 22 September 1997 ("052 application"), which was filed as a continuation of US application 08/781,752, filed on 10 January 1997 ("752 application"), which issued as U.S. Patent 5,945,577 on 31 August 1999.

3. Stice has been accorded the benefit for priority of the

[1] Stice exhibits are cited as SX 2___; Campbell exhibits are cited as CX 1___.

752 application. (Paper 1 at 3.)

4. According to Stice, its real party in interest is "the University of Massachusetts, which has exclusively licensed their interest to Advanced Cell Technology Corporation" (Paper 8 at 2).

5. Campbell is involved on the basis of application

09/989,126, filed 21 November 2001 (CX 1009),

as a continuation of

09/650,285, filed 29 August 2000 (now U.S. 6,525,243, issued 25 February 2003; CX 1031), as a continuation of

08/803,165, filed on 19 February 1997 (now U.S. 6,252,133, issued 26 June 2001; CX 1016), as the national stage of

PCT/GB96/02098, filed 30 August 1996 (CX 1013).

The PCT application is based on Great Britain application

GB 9517779.6, filed 31 August 1995 (CX 1010).

6. The specifications of Campbell's United States applications are said to be identical ('126 application, Paper 3a at 1).

7. Campbell has been accorded the benefit for priority with respect to the count of each of the applications cited in paragraph 5. (Paper 1 at 4.)

8. According to Campbell, its real party in interest is

(1) Assignee: Roslin Institute (Edinburgh) of Midlothian, England;

(2) licensees: Geron Corporation, of Menlo Park, CA,

and Exeter Life Sciences, Inc., of Phoenix, AZ.

(Paper 20 at 2.)

9. Terminal disclaimers were filed and accepted in Campbell's involved 126 application against any patent granted on:

application 09/989,128, filed 21 November 2001, (suspended);

application 09/989,125, filed 21 November 2001, (suspended); and U.S. Patent No. 6,252,133.

(126 application Paper 7, filed 11 April 2002.)

The count

10. Count 1, the sole count in this interference, reads:

Claim 1 Stice (6,235,970)

or

claim 20 Campbell (09/989,126).

11. The claims of the parties, all of which correspond to the count, are:

Stice: 1-21

Campbell: 20-36

12. Stice U.S. Patent No. 6,235,970, claim 1, reads:

A method for producing a mammalian cultured inner cell mass (CICM) cell line by nuclear transfer comprising the following steps:

(i) introducing a proliferating differentiated somatic mammalian donor cell or a proliferating differentiated somatic mammalian donor cell nucleus into an enucleated mammalian oocyte of the same species to produce a nuclear transfer unit;

(ii) activating the resultant nuclear transfer unit;

(iii) culturing said activated nuclear transfer unit until at least a size suitable for obtaining ICM cells;

(iv) isolating and culturing ICM cells obtained from said cultured nuclear transfer unit to obtain a cultured inner cell mass (CICM) or CICM cell line.

(Paper 9.)

13. Campbell 09/989,126, claim 20 reads:

A method for producing a mammalian cultured inner cell mass cell by nuclear transfer comprising:

(i) inserting a nucleus of a diploid non-human mammalian differentiated somatic cell in the G1 phase of the cell cycle into an unactivated, enucleated metaphase II-arrested non-human mammalian oocyte of the same species to reconstruct an embryo;

(ii) activating the resultant reconstructed embryo;

(iii) culturing said activated, reconstructed embryo; and

(v) isolating and culturing inner cell mass cells obtained from said cultured activated, reconstructed embryo to obtain a cultured inner cell mass cell.

(Paper 5.)

Preliminary Motions

Stice preliminary motions

14. Stice filed ten preliminary motions.

(1) Stice preliminary motion 1, to add its reissue application [10/833,993] to the interference. (Paper 18.)

(2) Stice preliminary motion 2, contingent on the grant of Stice preliminary motion 1, for benefit for priority of its 052 and 752 applications. (Paper 19.)

(3) Stice preliminary motion 3, to designate Stice claims 12 and 14 as not corresponding to the count. (Paper 31.)

(4) Stice preliminary motion 4, contingent on the grant of Stice preliminary motion 1, to designate Stice Reissue claim 14 as not corresponding to the count. (Paper 32.)

(5) Stice preliminary motion 5, to deny Campbell

benefit of priority of its GB application. (Paper 33.)

(6) Stice preliminary motion 6, to deny Campbell benefit of the Campbell PCT application. (Paper 34.)

(7) Stice preliminary motion 7, to designate Stice claims 19-21 and Campbell claims 31-36 as not corresponding to the count. (Paper 35.)

(8) Stice preliminary motion 8, for judgment that Campbell claims 20-30 are unpatentable under 35 U.S.C. § 112, ¶1, for lack of written description and lack of enablement for cultured inner cell mass cells. (Paper 36.)

(9) Stice preliminary motion 9, for judgment that Campbell claims 20-30 are unpatentable under 35 U.S.C. § 112, ¶2, because the claims allegedly are not drawn to Campbell's invention. (Paper 37.)

(10) Stice preliminary motion 10, contingent on the grant of Stice preliminary motion 1, to designate Stice reissue claims 19-21 and Campbell claims 31-36 as not corresponding to the count. (Paper 38.)

Campbell preliminary motions

15. Campbell filed five preliminary motions.

(1) Campbell preliminary motion 1, for judgment that Stice involved 970 patent lacks an adequate written description

of processes limited to a "proliferating" donor nucleus or cell. (Paper 24.)

(2) Campbell preliminary motion 2, for judgment that Stice involved claims are unpatentable under 35 U.S.C. § 112 ¶¶ 1 and 2, due to the lack of the allegedly critical limitation that the transplanted nuclei "can differentiate." (Paper 25.)

(3) Campbell preliminary motion 3, contingent on the grant of Campbell preliminary motion 2, to add claims to Campbell's involved specification. (Paper 26.)

(4) Campbell preliminary motion 4, contingent on the denial of Campbell preliminary motion 1, to substitute Count 2. (Paper 27.)

(5) Campbell preliminary motion 5, contingent on the grant of Campbell preliminary motion 4, for benefit of the GB application for proposed Count 2. (Paper 28.)

III. Discussion

Campbell Motion 1

16. Campbell seeks judgment that Stice's involved claims 1-21 are unpatentable for lack of an adequate written description of the limitation that the donor cell or nucleus is a proliferating donor cell or nucleus. (Paper 24 at 6.)

17. Stice claim 1 is representative (bold added):

> A method for producing a mammalian cultured inner cell mass (CICM) cell line by nuclear transfer comprising the following steps:
> (i) inserting a **proliferating differentiated somatic mammalian donor cell** or a **proliferating differentiated somatic mammalian donor cell nucleus** into an enucleated mammalian oocyte of the same species to produce a nuclear transfer (NT) unit;
> (ii) activating the resultant nuclear transfer unit;
> (iii) culturing said activated nuclear transfer unit until at least a size suitable for obtaining ICM cells;
> (iv) isolating and culturing ICM cells obtained from said cultured nuclear transfer unit to obtain a cultured inner cell mass (CICM) or CICM cell line.

(The other independent claim, claim 19, contains a similar limitation, viz., "wherein the donor cell is a **proliferating** mammalian differentiated cell or wherein the donor nucleus is from a **proliferating** mammalian differentiated cell." (Paper 9 at 4; bold added).)

18. Neither party disputes that the plain language of the Stice claims indicates that the donor cell or donor cell nucleus must be "proliferating."

19. Campbell urges that the Stice specification does not describe, directly or indirectly, processes that contain this specific limitation:

> Although the '970 patent specification uses the term 'propagating' or 'propagated' in reference to colonies or cultures of cells (CX 1001 at col. 16, lines 33-36 and 56), this term is only used in the context of

> growing populations of cells, which would contain both proliferating and non-proliferating cells. The term is never used in the context of the cell cycle status of a particular cell being used for nuclear transfer as required by Stice's involved claims.

(Paper 24 at 14.)

20. Campbell restates the problem in its reply: "[t]he issue is whether the disclosure of a propagating population of cells, which contains both propagating and non-propagating cells, supports a claim that requires the selection of a single propagating cell from the population." (Campbell reply 1, Paper 64 at 3.)

21. Campbell relies on testimony by Dr. David Wells, Ph.D. ("Wells") (Paper 24 at 4-6 and 15-17).

22. We find that Wells is qualified as an expert witness in the areas of cloning livestock animals, cell cultures, and nuclear transplantation. (CX 1007.)

23. Wells testifies that propagating (proliferating) cells are recognized as being in one of the four stages of the "cell cycle" (G_1, S_1, G_2, M); whereas "quiescent" (living but non-propagating) cells are recognized as being in the "G_0" stage, i.e., as not being in any stage of the cell cycle. (CX 1007 at 2.)

24. Wells testifies further that "[b]ased on my experience using cultured proliferating mammalian differentiated somatic

cells in various stages of the mitotic cell cycle, cell cultures contain cells at various stages of the cell cycle, including cells that are in the G_0 stage and which are therefore not proliferating." (CX 1007 at 3, ¶ 6.)

25. Wells testifies further that the article by Boquest[2] shows that approximately 2.8% of the cells in a cycling (i.e., proliferating) porcine fibroblast cell population were in the G_0 phase of the cell cycle. (CX 1007 at 4, citing CX 1006 at 1016, Table 1.)

26. According to Wells, Boquest reports still higher amounts of G_0 cells in populations of cycling cells that were "grown to confluency." (CX 1007 at 4, citing CX 1006 at 1016, Table 1.)

27. Wells concludes that a propagating culture of fibroblast cells contains both proliferating and quiescent cells. (CX 1007 at 4, ¶ 16.)

28. Campbell urges that Stice's involved specification does not inherently describe transplanting a proliferating cell or a proliferating nucleus into an enucleated oocyte because taking a cell from a proliferating culture does not necessarily result in taking a proliferating cell from that culture. (Paper 24 at 15.)

---

[2] A.C. Boquest et al., *Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells*, 60 BIOL. REPROD. 1013 (1999) (CX 1006).

29. Stice, in contrast, urges that the "proliferating" limitation is inherent in Example 1 of its involved specification, which describes taking a cell from a clonal fibroblast cell line propagated in a growth medium containing 10% fetal calf serum." (Stice opposition 1, Paper 46 at 8; Stice contingent opposition 1, Paper 47, is moot in view of our denial of Stice preliminary motion 1, *infra*).

30. Stice relies on the testimony of Michael D. West, Ph.D. (Paper 46 at 8-9.)

31. West describes himself as the President, Chief Executive Officer, and Chairman of Advanced Cell Technology, Inc. ("ATC"). (SX 2009 at 2, ¶ 1.)

32. ATC holds an exclusive license to the technology developed by Stice that is involved in this interference. (Fact 4, *supra*.)

33. West has published technical articles in the area of telomerase activity, cell senescence, stem cells and cloning. (SX 2008.)

34. We find that West is qualified to testify as an expert in this interference.

35. West testifies:

> 10. Based on my experience with cell culture, fibroblasts propagated in growth medium containing 10% fetal calf serum and not intentionally grown to very high levels of confluence over an extended period of time would be proliferating, not quiescent.
>
> 11. Based on my experience with cell culture, the propagation of fibroblasts requires splitting and transferring propagating cells into fresh growth medium containing 10% fetal calf serum. The process of diluting and splitting propagating cells during, for example, propagation of colonies of cells from single cells, dilutes out senescent, quiescent and non-propagating cells until the cells have proliferated to the point of senescence.

(SX 2025 at 3.)

36. West quotes from the Stice involved specification,

Example 1:

> Fibroblast cells were plated in tissue culture dishes and cultured in alpha-MEM medium (Bio Whittaker, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, Utah) . . . Each colony was propagated independently of each other . . . One line of cells (CL-1) derived from one colony of bovine embryonic fibroblast cells was used as donor nuclei in the nuclear transfer (NT) procedure.

Column 16, lines 7-11, 49, and 57-59 of the '970 specification.[3]

(SX 2025 at 4, ¶ 12.)

---

[3] We find the cited text from Stice Example 1 at column 16, lines 14-18, 56, and 64-66. (SX 2002 at col. 16.)

37. West concludes that "Stice clearly demonstrated use of propagating, synonymous with proliferating fibroblasts for nuclear transfer throughout [the] '970 specification." (SX 2025 at 4, ¶ 14.)

38. Stice does not comment on the Boquest article or on Wells' testimony regarding the Boquest article.

Discussion

Whether claimed subject matter is described by the specification within the meaning of 35 U.S.C. § 112, first paragraph, is a question of fact. *E.g., Noelle v. Lederman*, 355 F.3d 1343, 1348, 69 USPQ2d 1508, 1513 (Fed. Cir. 2004). It is Campbell's burden to come forward with a preponderance of the evidence that the claimed invention is not adequately described. 37 CFR § 41.121(b) (effective 13 September 2004); 37 CFR § 1.637(a) (2003); *Velander v. Garner*, 348 F.3d 1359, 1369-70, 68 USPQ2d 1769, 1777 (Fed. Cir. 2003). A party that seeks to prove that a certain limitation is inherent, however, may not rely on probabilities or possibilities. *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1951 (Fed. Cir. 1999) ("To establish inherency, the extrinsic evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference . . . Inherency, however, may not be established by probabilities or possibilities. The mere

fact that a certain thing may result from a given set of circumstances is not sufficient.") (internal quotes and citations omitted).

The parties agree that the disputed limitation is not expressly present in Stice's involved specification. We find that Campbell, through the testimony of Wells, has established a sound factual basis for doubting that every cell taken from a proliferating cell culture such as described in Stice Example 1 is necessarily a proliferating cell. The weight of the evidence shows that the selected cell may not always be proliferating; in other words, it may be quiescent. Wells' testimony is supported by the Boquest article, which appears to be entirely independent of party Campbell. We find the testimony of West, in support of Stice, to be unpersuasive, as it is essentially conclusory. Not only is West's testimony not supported by an independent publication, but West fails to explain how the procedures described in Stice Example 1 relate to the "process of diluting and splitting propagating cells" that is said to "dilute out" senescent, quiescent, and non-propagating cells from the culture. First, West's statement indicates that non-propagating cells are present to some extent in such cultures, at least initially, thus confirming Campbell's predicate that nonpropagating cells are present in at least some cultures of propagating cells. Moreover

West does not testify that the processes employed in Stice Example 1 invariably and inevitably result in samples that contain no nonpropagating cells. West has failed to explain the underlying technical basis of his opinion, and we do not accord West's testimony significant weight.

We find that a preponderance of the evidence shows that Stice's general descriptions and its specific examples do not amount to an inherent description of processes wherein propagating cells or the nuclei of propagating cells are implanted into enucleated oocytes. Accordingly, Campbell preliminary motion 1 is GRANTED.[4]

Campbell preliminary motion 4, which is contingent on the denial of Campbell preliminary motion 1, is DISMISSED as moot.

Campbell preliminary motion 5, which is effectively contingent on the grant of Campbell preliminary motion 4, is DISMISSED as moot.

Stice motion 3, to designate certain Stice claims as not corresponding to the count is DISMISSED as moot because we have found all of Stice's claims to be unpatentable on grounds unrelated to the basis of Stice motion 3.

---

[4] The Board's decision in Interference 104,746 (Paper 80 of that proceeding; CX 1003) is based on a different patent and a distinct record. Therefore, it is not controlling or binding on this panel. We decide each case on its own facts and merits, as it comes before us.

Stice motion 7, to designate certain Stice and Campbell claims as not corresponding to the count is DISMISSED as moot because we have found all of Stice's claims to be unpatentable on grounds unrelated to the basis of Stice motion 7.

Campbell motion 2

Having determined that none of the Stice involved claims are patentable for lack of an adequate written description, we need not consider Campbell preliminary motion 2, that the claims are unpatentable for additional reasons.

Accordingly, Campbell preliminary motion 2 is DISMISSED.

Campbell preliminary motion 3, which is contingent on the grant of Campbell preliminary motion 2, is DISMISSED as moot.

Stice Motion 1

39. Stice preliminary motion 1 seeks to add its reissue application, 10/833,993, filed 28 April 2004, which is based on its involved 970 patent to the interference. (Paper 18.)

40. Specifically, Stice seeks to "replace present claims 1-21 of U.S. Patent 6,235,970 as corresponding to the count of the present interference and that claim 1 of the reissue application as amended corresponds to count 1 of the present interference." (Paper 18 at 2.)

41. Stice states that, in the reissue application, "claims 1, 19, and claim 14 made independent, have been amended to delete the term 'proliferating' and to substitute the term 'propagating.'" (Paper 18 at 3.)

42. Thus, Stice is presenting a reissue application that contains only new claims.

43. Stice has canceled claim 12 of the reissue application. (SX 2001 at 3.)

44. Campbell points out that Stice's reissue claim 1 omits a number of terms, but addressed itself to the substantive issues raised as if the claims contained the missing terms. (Campbell opposition 1, Paper 53 at 1.)

45. Stice, in reply, urges that it has submitted an amendment correcting the omissions. (Stice reply 1, Paper 71 at 2-3.)

46. Review of the official record (i.e., the electronic file) of reissue application 10/833,993, shows that no such amendment was present in the file.

47. Stice preliminary motion 4, which is contingent on the grant of Stice preliminary motion 1, seeks to designate claim 14 of its reissue application as not corresponding to the count. (Paper 32.)

48. Pursuant to a conference call by the Board on December 28, 2004, Stice filed a miscellaneous motion (hereafter, "Stice motion 11") to amend its reissue application 10/833,993 by canceling claim 14 and amending claim 1 to correct inadvertent word-processing errors (Paper 92 (Stice motion 11) and SX 2033 (proposed amendment)).

Discussion

Trial Section precedent interprets Rule 633(h) (July 1, 2004) "to permit the filing of a preliminary motion to add a reissue only if the reissue applicant agrees that all 'new' claims in the reissue application are to be designated as corresponding to the count." *Winter v. Fujita*, 53 USPQ2d 1478, 1483 (Bd. Pat. App. & Int. 2000). Stice preliminary motion 4 indicates that Stice regards claim 14 as not corresponding to the count, but Stice motion 11 removes this obstacle to considering Stice preliminary motion 1.

Ordinarily, an application, including a reissue application will not be placed into an interference unless the claims have been indicated to be allowable by a Primary Examiner.[5] In

---

[5] *Cf. Brenner v. Manson*, 383 U.S. 519, 528 n.12, 148 USPQ 689, 693 n.12 (1966):

> "[t]here is no basis for the proposition that even where an applicant for an interference presents a claim which on its face is unpatentable, a complicated and frequently lengthy factual inquiry into priority of invention must inexorably take place. On the contrary, Rule 201(a), 37 CFR § 1.201(a), defines an interference proceeding as one involving "two or more parties claiming

extraordinary circumstances, however, it may be appropriate to exercise, on the behalf of the Director of the United States Patent and Trademark Office, discretion to resolve issues efficiently despite procedural irregularities. This is such a case. Accordingly, we GRANT Stice preliminary motion 1 to add reissue application 10/833,993 to this interference.

As a consequence of adding the reissue application, we must consider whether the claims of the reissue application are patentable to Stice. For the following reasons, we conclude that they are not. The differences between claim 1 of the Stice involved 970 patent and proposed reissue claim 1 are the substitutions of the word —propagating— for each occurrence of the word "proliferating" in step (i) of the claimed process. Step (i) of Reissue application claim 1 reads:

> (i) introducing a [proliferating] propagating differentiated somatic mammalian donor cell or a [proliferating] propagating differentiated somatic mammalian donor cell nucleus into an enucleated

---

substantially the same patentable invention and may be instituted as soon as it is determined that common patentable subject matter is claimed * * *." (Emphasis supplied.) See *Application of Rogoff*, 46 CCPA 733, 739, 261 F.2d 601, 606, 120 USPQ 185, 188: "The question as to patentability of claims to an applicant must be determined before any question of interference arises and claims otherwise unpatentable to an applicant cannot be allowed merely in order to set up an interference."

mammalian oocyte of the same species to produce a nuclear transfer unit;

in which square brackets indicate text deleted from the patent claim and the underscores indicate text added to the patent claim. (SX 2002 at col. 18 and SX 2033 at 4.) Stice indicated in its principal brief and at oral argument that it regards the terms "propagating" and "proliferating" as having the same meaning. (Paper 18 at 7; Paper 91 at 12, ll. 10-16.) The difference, according to Stice, is that the word "propagating" is present in the Stice specification, whereas "proliferating" is not. (Paper 91 at 12, ll. 16-18.)

We held *supra* in our decision on Campbell motion 1 that the involved Stice specification lacks an adequate written description of the particular subgenus of processes in which the donor cell or the donor cell nucleus is itself "proliferating." On the present record, the description of processes in which a cell is taken from a culture that contains proliferating cells, without more, is not necessarily and inevitably a description of a process in which the selected and transferred cell or nucleus is "proliferating." The reason is that the cell taken from the proliferating culture is not necessarily and inevitably a proliferating cell – it may be a quiescent cell. Merely using the word "propagating," which does occur in the specification,

instead of "proliferating," which does not, does not change the failure of the originally filed specification to describe the invention now claimed.

We therefore find that the claims of Stice's reissue application, as amended, are not patentable to Stice for lack of an adequate written description of the claimed process.

Stice preliminary motions 2, 4, and 10, which are contingent both on the grant of Stice preliminary motion 1 and on the implicit assumption that such claims will be found patentable to Stice, are DISMISSED as moot.[6]

Stice motion 8

Stice preliminary motion 8 seeks, pursuant to 37 CFR § 1.633(a), judgment that Campbell's involved application provides neither an adequate written description nor an enabling disclosure for Campbell's involved claims 20-30. In contrast to Stice motions 5 and 6, Stice bears the burden of showing that Campbell's involved application does not describe or enable the full scope of Campbell's involved claims within the meaning of 35 U.S.C. § 112, first paragraph. Although Stice describes Campbell

[6] Stice reissue claim 14 is related to human embryos because it involves creating a reconstructed human embryo and then producing CICM cell line from the ICM cells of said human embryo. (SX 2001 at 3.) Because we have determined that this claim is not properly before us on other grounds, we need not address the question of whether the USPTO has authority to consider the patentability of this claim. The Consolidated Appropriations Act, 2005, HR4818, Title VI, § 626 reads, "None of the funds appropriated or otherwise made available under this Act may be used to issue patents on claims directed to or encompassing a human organism."

dependent claims 21-30 in its brief (Paper 36 at 19-21, facts 15-24), we discern no separate argument against the patentability of these claims. Accordingly, we restrict our attention to Stice's case for the inadequacy of support for claim 20.

Regarding written description, Stice contends in its principal brief that the Campbell involved 126 specification lacks any description of culturing inner cell mass cells. (Paper 36 at 22.) For the reasons discussed in detail *infra*, we reject this argument as factually incorrect.

Campbell claim 20 reads:

> A method for producing a mammalian cultured inner cell mass cell by nuclear transfer comprising:
> (i) inserting a nucleus of a diploid non-human mammalian differentiated somatic cell in the G1 phase of the cell cycle into an unactivated, enucleated metaphase II-arrested non-human mammalian oocyte of the same species to reconstruct an embryo;
> (ii) activating the resultant reconstructed embryo;
> (iii) culturing said activated, reconstructed embryo; and
> (iv) isolating and culturing inner cell mass cells obtained from said cultured activated, reconstructed embryo to obtain a cultured inner cell mass cell.

Campbell's involved 126 specification contains the following description:

> According to a third aspect of the invention, there is provided a method of preparing an animal, the method comprising:

(a) reconstituting an animal embryo as described above; and
(b) causing an animal to develop to term from the embryo; and
(c) optionally, breeding from the animal so formed.

Step (a) has been described in depth above.

The second step, step (b) in the method of this aspect of the invention is to cause an animal to develop to term from the embryo. This may be done directly or indirectly. . . . In indirect development, however, the embryo may be further manipulated before full development takes place. For example, the embryo may be split and the cells clonally expanded, for the purpose of improving yield.

Alternatively or additionally, it may be possible for increased yields of viable embryos to be achieved by means of the present invention by clonal expansion of donors and/or if use is made of the process of serial (nuclear) transfer. A limitation in the presently achieved rate of blastocyst formation may be due to the fact that a majority of the embryos do not "reprogram" (although an acceptable number do). If this is the case, then the rate may be enhanced as follows. Each embryo that does develop itself can be used as a nuclear donor at the 32-64 cell stage; alternatively, inner cell mass cells can be used at the blastocyst stage. If these embryos do indeed reflect those which have reprogrammed (as seems likely), then each developing embryo may be multiplied in this way by the efficiency of the nuclear transfer process. The degree of enhancement likely to be achieved depends upon the cell type.

(CX 1009 at 15-16 (Campbell involved 126 application); see also CX 1010 at 15-16 (GB application); CX 1013 at 15-16 (PCT application); underscore added.)

We find that Campbell in this passage refers to a process in which an embryo is reconstructed by nuclear transfer into an enucleated oocyte (step (a)). From Campbell's prior description of step (a), we understand (and Stice does not dispute) that Campbell teaches steps (i), (ii), and (iii) of the process covered by claim 20. Regarding step (b), we find that Campbell teaches that the reconstructed embryo may be subjected to indirect development. For example, cells from the embryo may be clonally expanded, "for the purpose of improving yield." Wells testified, "[b]ased on my experience in clonally expanding embryonic cells, in the context of the 126 application, 'clonally expanding' embryonic cells means generating daughter cells by cell division using *in vitro* cell culture of embryonic cells." (CX 1033 at 11-12, ¶ 56.) Thus, we understand from Wells' testimony that clonal expansion of cells involves removing and isolating cells from the embryo and then culturing the cells.

In the first two underscored passages, Campbell teaches generally that daughter cells may be generated by cell division in a culture. In the third underscored passage, Campbell teaches that the source of embryonic cells to be expanded can be ICM cells from the blastocyst stage of the embryo. Such expansion involves culturing the ICM cells. Thus, we find that Campbell provides an adequate description of a process of reconstituting

an embryo by nuclear transplantation comprising steps (i), (ii), (iii), and further, isolating and culturing the ICM cells of the embryo as required by step (iv) of the process recited in Campbell claim 20. In other words, we find that Campbell describes a process in which a reconstructed embryo is created, ICM cells are harvested from the blastocyst stage of the reconstructed embryo, and the ICM cells are then clonally expanded. That description supports Campbell claim 20 within the meaning of 35 U.S.C. § 112, first paragraph.

Stice urges a new theory in its Reply (Paper 78 at 3-4), that Campbell's involved claims lack the allegedly critical limitation that Campbell teaches culturing ICM cells only for the purpose of nuclear transplantation. Stice argues that Campbell is not entitled to broad claim 20, which does not have any use limitations. (Paper 78 at 4.) We dismiss Stice's new ground of argument because it is belated. Stice has not offered any reason that it could not have raised its new thesis in its principal brief. However, even if we were to entertain Stice's new argument, we would reject it to the extent that Stice's rebuttal argument urges that Campbell failed to describe a useful invention commensurate in scope with Campbell claim 20. Stice's reliance on *Gentry Gallery, Inc. v. Berkline Corp.*, 134 F.3d 1473, 1479, 45 USPQ2d 1498, 1503 (Fed. Cir. 1998), is inapposite,

as Stice has failed to direct our attention to any disclosure in Campbell's 126 specification that indicates that there are no purposes other than nuclear transplantation for which the cultured ICM cells would be useful. Nor has Stice come forward with any probative evidence, e.g., expert testimony or technical articles, that indicate that no uses for such cells were recognized.

As for the alleged lack of enabling disclosure in Campbell's application, Stice argues that "[t]he '126 specification does not disclose any methods for making and using the cultured inner cell mass cells of Claims 20-30." (Paper 36 at 23.) Stice also argues that "Campbell's '126 specification does not disclose any examples, prophetic or working, describing isolation, preparation, and culturing of cultured inner mass cells." (Id.) Stice concludes that it would have required undue experimentation to make and use the cultured inner cell mass cells of claims 20-30. (Id. at 23-34.)

Stice's lack of enablement argument is not persuasive because Stice has not established via expert testimony and citation to relevant scientific and technical literature that one skilled in the art would not have been able to carry out the described embodiment without undue experimentation. Although it was not required to do so, Campbell came forward with evidence

that the culturing of cells, including the culturing of ICM cells, was within the ordinary skill of the art. With regard to enablement, Campbell cites scientific publications and testimony by Wells, indicating that culturing cells, including ICM cells, was a well-established technology prior to the 1995 filing date of the GB application. (Paper 57 at 15-16; Paper 58 at 15-16.) Stice has not put forward any credible evidence to the contrary.

In conclusion, Stice has failed to come forward with sufficient probative evidence to establish that the absence of disclosure in Campbell's involved 126 application would require undue experimentation on the part of the ordinary worker to make and use the claimed invention.

Stice preliminary motion 8 is DENIED.

Stice motions 5 and 6

Stice motion 5 seeks, pursuant to 37 CFR § 1.633(g), to deny Campbell the benefit for priority accorded to Campbell's GB application. (Paper 33 at 2.) Stice motion 6 seeks, pursuant to 37 CFR § 1.633(g), to deny Campbell the benefit for priority accorded to Campbell's PCT application. (Paper 34 at 2.) Stice concedes that the texts of the GB disclosure and the PCT disclosure are substantially the same as the text of Campbell's involved 126 application. (Paper 33 at 11; Paper 34 at 10.) In

both motions 5 and 6, Stice asserts that the benefit of priority requires that the earlier application must provide "an enabling disclosure and written description of the invention claimed in the U.S. application." (Paper 33 at 10; Paper 34 at 10.) Stice urges that Campbell's GB and PCT applications fail in both regards. (*Id.*) Stice, however, limits its arguments to the alleged lack of written description. We have not found any substantive argument directed to the alleged lack of enablement.

In each motion, Stice presents a table which recites, in the first column, the text of Campbell claim 20. The second column of the table recites the support in the PCT and GB ("UK") application allegedly cited by Campbell to the examiner in support of that claim. The third column of the table, which is labeled "Analysis," states that the support recited in column 2 "[d]oes not describe or define what is meant by 'cultured inner cell mass cell.'" (Paper 33 at 11-21; Paper 34 at 10-21.)

Stice next urges that benefit for priority is accorded if the "UK" or PCT application is a constructive reduction to practice, which is satisfied if the application in question satisfies the requirements of 35 U.S.C. § 112, first paragraph, "with at least one embodiment of the count." (Paper 33 at 21 and Paper 34 at 21, each citing *Hunt v. Treppschuh*, 523 F.2d 1386, 1389, 187 USPQ 426, 429 (CCPA 1974).) Stice then urges that:

> [w]e have shown in the previous section that Campbell's [UK/PCT] application does not comply with 35 USC § 112, first paragraph, due to lack of support for Claim 20, designated as the Count. Correspondingly, Campbell's [UK/PCT] application does not fulfill this requirement because it does not provide support within the specification as described herein for the count of a method for producing [a] cultured inner cell mass cell or an inner cell mass cell line, which includes Stice Claim 1 as the other option.

Stice appears to have conflated the requirements for obtaining the benefit for priority in an interference with the requirements for securing the benefits of a prior filing date under 35 U.S.C. §§ 119 and 120. Benefit for priority in an interference is established by demonstrating that a single embodiment within the scope of the count is described and enabled within the meaning of 35 U.S.C. § 112, first paragraph. Such an embodiment, if prior to the earliest benefit date of an opposing party, would preclude, under 35 U.S.C. § 102(g), the grant of a patent to the opposing party. That is, the earlier disclosure would be evidence of prior invention, by another, of the commonly claimed subject matter. Benefit under §§ 119 and 120, however, requires that the entire scope of the claim be both described and enabled. The scope of proof required under §§ 119 and 120 is broader because the benefit precludes the application of art that would otherwise deny patentability of the claimed subject matter.

Stice's arguments are unsatisfactory both procedurally and substantively. Stice fails to make a *prima facie* case that Campbell's specification lacks an adequate written description or an enabling disclosure of the claimed subject matter. Stice's allegations and arguments are not supported by convincing expert testimony that explains what one skilled in the art would understand Campbell's specification to teach. Stice's arguments in its Reply are untimely, as there is no apparent reason why they were not presented in its principal brief.

Substantively, we found in regard to Stice motion 8, *supra*, that the same substantive disclosure in Campbell's involved specification satisfied the heavier burden of written description and enablement of the full scope of the same subject matter. Thus, we readily determine that the lighter burden of description and enablement of a single embodiment within the scope of the count, as defined in the alternative by Campbell claim 20, is fully met.

We conclude that Stice has not carried its burden of proof that Campbell's GB and PCT applications lack a written description or an enabling disclosure of at least one embodiment of a process within the scope of the count. Accordingly, Stice preliminary motions 5 and 6 are DENIED.

Stice preliminary Motion 9

Stice moves for judgment that Campbell claims 20-30 are unpatentable under 35 U.S.C. § 112, second paragraph. (Paper 37 at 2.) Stice urges that Campbell claims 20-30 "do not set forth essential elements of what is regarded by the inventors to be the invention." In Stice's view, the essential elements of Campbell's invention comprise embryos that are "capable of giving rise to a live birth." (Paper 37 at 22, emphasis not reproduced.) At best, the cases cited by Stice support its argument only in *dicta*. Although Stice has cited many excerpts from Campbell's specification, it has not directed our attention to any positive statements that certain intended uses are "essential elements" of the invention. Stice has also failed to identify any statements in the Campbell specification that certain uses, if not recited in a claim, disqualify the invention so defined from being Campbell's invention. Nor has Stice pointed to any such statements in the prosecution history. We decline to hold claims indefinite on the slim predicate Stice infers from its interpretation of Campbell's specification.

Stice urges further that the claims are indefinite in the term "cultured inner cell mass cell," because that term is not defined in Campbell's specification, and it would not have had an apparent meaning based on the prior art. (Paper 37 at 24, citing

the second declaration of Michael D. West ("West 2"), SX 2010.) The argument is belied by West 2 (SX 2010 at 4, ¶ 4), which cites an article, Michelle Sims and N.L. First, *Production of calves by transfer of nuclei from* ***cultured inner cell mass cells***, 90 PROC. NAT'L. ACAD. SCI. USA 6143 (1994) (bold emphasis added). West testified that the knowledgeable person in the art understood distinctions between "non-cultured ICM cells" and "cultured ICM cells." (SX 2010 at 4, ¶ 4.) Thus, we find the factual basis of Stice's argument to be incorrect.

Stice preliminary motion 9 is DENIED.

IV. Order

In view of the foregoing considerations, it is:

ORDERED that Stice preliminary Motion 1 to add reissue application 10/833,993 is GRANTED.

FURTHER ORDERED that Stice Reissue application 10/833,993 is added to this interference.

FURTHER ORDERED that claims 1-11, 13, and 15-21 of Stice Reissue application 10/833,993 are not patentable to Stice under 35 U.S.C. § 112, first paragraph, for lack of written description.

FURTHER ORDERED that Stice preliminary motions 2, 4, and 10, which are contingent on the grant of Stice preliminary

motion 1 and the holding that the claims of Reissue application 10/833,993 are patentable to Stice, are DISMISSED.

FURTHER ORDERED that Stice preliminary 5, 6, and 8 are DENIED.

FURTHER ORDERED that Stice Motions 3 and 7 are DISMISSED.

FURTHER ORDERED that Stice preliminary motion 9 is DENIED.

FURTHER ORDERED that Campbell preliminary motion 1 is GRANTED.

FURTHER ORDERED that Campbell preliminary motions 2, 3, 4, and 5 are DISMISSED as moot.

FURTHER ORDERED that this paper be given an appropriate number and placed in the patent file of U.S. Patent 6,235,970, in the application file of 09/989,126, and in the reissue application file of 10/833,993.

FURTHER ORDERED that if there is a settlement, the attentions of the parties are directed to 35 U.S.C. § 135(c) and 37 CFR § 41.205.

FURTHER ORDERED that attention is directed to the Judgment issued in accompanying Paper 95.

FRED E. McKELVEY
Senior Administrative Patent Judge

SALLY GARDNER LANE
Administrative Patent Judge

MARK NAGUMO
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TRIAL SECTION MERITS PANEL

Alexandria, VA
11 February 2005

cc: via overnight mail:

Counsel for Stice:

Ronald A. Daignault, Esq.
MERCHANT & GOULD, P.C.
1101 30th Street, N.W.,
Suite 500
Washington, DC 20007

Phone: 202-625-8380
Fax: 202-625-8381

Counsel for Campbell:

Kenneth J. Meyers, Esq.
FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, LLP
1300 I Street, N.W.,
Suite 700
Washington, DC 20005

Phone: 202-408-4000
Fax: 202-408-4400

The opinion in support of the decision being entered today is not binding precedent of the Board.

Paper 94

Filed by: Trial Section Merits Panel
Mail Stop Interference
P.O. Box 1450
Alexandria, VA 22313-1450
Tel: 571-272-9797
Fax: 571-273-0042

Filed
11 February 2005

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES
(Administrative Patent Judge Mark Nagumo)

STEVEN L. **STICE**,
JOSE CIBELLI, JAMES ROBL,
PAUL GOLUEKE, F. ABEL PONCE de LEON
and D. JOSEPH JERRY,

Junior Party,
(Patent 6,235,970 and
Reissue Application 10/833,993),

v.

KEITH HENRY STOCKMAN **CAMPBELL**
and IAN WILMUT,

Senior Party,
(Application 09/989,126).

Patent Interference No. 105,192

Before: MCKELVEY, Senior Administrative Patent Judge, LANE, and NAGUMO, Administrative Patent Judges.

NAGUMO, Administrative Patent Judge.

JUDGMENT

I. Introduction

The following findings of fact are supported by a preponderance of evidence in the record.

1. As a result of the findings of fact and conclusions of law set out in Paper 93 (Decision - substantive motions) of this interference, Stice is not entitled to a patent to any claims of its involved U.S. patent No. 6,235,970.

2. As a result of the findings of fact and conclusions of law set out in Paper 93 (Decision - substantive motions) of this interference, Stice is not entitled to a patent to any claims of Stice reissue application 10/833,993, which is based on the Stice 6,235,970 patent.

III. Discussion

An interference is a proceeding to determine whether or not a patent may be issued to an applicant based on an application, all the claims of which are allowable but for the possibility that another first invented the same subject matter. 35 U.S.C. § 102(g). *Cf. Case v. CPC Int'l, Inc.*, 730 F.2d 745, 750, 221 USPQ 196, 200 (Fed. Cir. 1984) ("[n]o interference in fact means that there is no interfering subject matter, that Case's patent is no impediment to granting CPC the claims of its application.")

Stice is not entitled to any of its patented claims

corresponding to the count: thus, Stice patent 6,235,970 is not an impediment to the issuance of a patent to Campbell based on the 09/989,126 involved application. Similarly, Stice is not entitled to a patent on any of the claims in its reissue application: Thus, the Stice reissue application is not an impediment to the issuance of a patent to Campbell based on the 10/833,993 reissue application. Moreover, Campbell, as the senior party, is presumed to be entitled to the decision on priority.

Under these circumstances, no purpose would be served by proceeding to a priority contest in this interference.

II. Order

In view of the findings of fact and conclusions of law set out in Paper 93 (Decision - substantive motions) of this interference, it is:

ORDERED that Steven L. Stice, Jose Cibelli, James Robl, Paul Golueke, F. Abel Ponce de Leon, and D. Joseph Jerry are not entitled to a patent containing claims 1-21 of U.S. Patent No. 6,235,970.

FURTHER ORDERED that Steven L. Stice, Jose Cibelli, James Robl, Paul Golueke, F. Abel Ponce de Leon, and D. Joseph

Jerry are not entitled to a patent on claims 1-11, 13 and 15-21 of reissue application 10/833,993.

FURTHER ORDERED that this judgment is final for purposes of appeal regarding the status of Stice's 6,235,970 patent.

FURTHER ORDERED that this paper be given an appropriate number and placed in the patent file of U.S. Patent 6,235,970, in the application file of 09/989,126, and in the reissue application file of 10/833,993.

FURTHER ORDERED that the reissue application is returned to the jurisdiction of the primary examiner for action not inconsistent with this decision.

FURTHER ORDERED that the attention of Campbell and the primary examiner is directed to related cases 09/989,178 and 09/989,125, both currently suspended;

FURTHER ORDERED that if there is a settlement, the attentions of the parties are directed to 35 U.S.C. § 135(c) and 37 CFR § 41.205.

FRED E. McKELVEY
Senior Administrative Patent Judge

SALLY GARDNER LANE
Administrative Patent Judge

MARK NAGUMO
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TRIAL SECTION MERITS PANEL

Alexandria, VA
11 February 2005

cc: via first class mail:

Counsel for Stice:

Ronald A. Daignault, Esq.
MERCHANT & GOULD, P.C.
1101 30th Street, N.W.,
Suite 500
Washington, DC 20007

Phone: 202-625-8380
Fax: 202-625-8381

Counsel for Campbell:

Kenneth J. Meyers, Esq.
FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, LLP
1300 I Street, N.W.,
Suite 700
Washington, DC 20005

Phone: 202-408-4000
Fax: 202-408-4400